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More ups and downs for US science

Like the Grand Old Duke of York (the one who marched his men to the top of the hill and then down again), President Carter seems bent on making those who depend on his administration for financial support survive successive spells of euphoria and depression. This, at least, is how the chopping and changing on the budget for the coming year (which starts at the beginning of next month) must seem. The tale, chronicled at length in the past few months, is especially bewildering for the research community, which is aware both of the need to be able to plan ahead and of the attempts by Congress (at the urging of the Administration) to devise some way in which the basic research budget can be made less vulnerable to annually changing fashions.

The tale goes like this. In January, the President's budget request to the Congress, in aggregate unbalanced and therefore a disappointment to those who had taken Mr Carter's election promises seriously and also disappointing to foreign exchange dealers, provided for a substantial growth of real support for research and development. The total cost of research and development to federal agencies was to grow rather more quickly than inflation, but basic research was to be treated even more generously with an extra 3 per cent in real terms between the two financial years. The word went out that these provisions were a measure of the Administration's commitment to the importance of basic research. Spirits soared at the National Science Foundation and the National Institutes of Health, which were to be dealt with especially generously.

These spirits did not last for long. Before Congress had time to buckle down properly to its annual and needlessly tedious scrutiny of the proposals, economic crisis was the order of the day. With the prospect of increased inflation, people took to borrowing from the banks to buy goods on tick, with the result that the money supply increased dramatically. Professor Milton Friedman's followers were no doubt kept awake at night with worry, and the dollar slumped on the international exchanges. So the White House itself took a knife to the budget. In total, the discretionary parts of the budget were reduced by some 8 per cent, credit was restricted and interest rates raised. The word went out that these steps were a proof of the Administration's commitment to the battle against inflation. Fair play, research and development did relatively well. Basic research was especially well favoured, but the prospect of growth melted away. Some were brave enough to suggest that this relative leniency was a proof of the Administration's belief in the importance of research and development.

Now the troops are back at the top of the hill again (see *Nature*, 4 September), or are at least half way there. At the end of last month, President Carter announced his "Economic Program for the Eighties" which is a complicated mixture of tax cuts, incentives to further industrial investment, provision for helping people adjust to rapidly changing economic circumstances and — here comes the surprise — more money for research and development, especially for basic research. For practical purposes, the new proposals put the science budget where it was in January, but also give Dr Frank Press, the President's Science Advisor, an extra \$255 million to allocate to new projects. Moreover, the President has said that there will be a further increase of 3 per cent on basic research expenditure in the following financial year (beginning on 1 October 1981). So, predictably, the word has gone out that the Administration's commitment to the importance of research and development, and

to basic research in particular, has been demonstrated once again. The only fly in the ointment is that much of this promise will not become reality until next January, when President Carter hopes to introduce his next budget resolution to Congress. There is as yet no sign of what Mr Reagan would do if he had the responsibility.

Several questions arise from this curious tale, not the least of which is whether the Administration is really serious in its protestations that the interests of research and development, particularly of basic research, lie close to its heart. The issue is complicated because the President's motives for the timing of his announcement of the new programme of economic recovery are dubious, to say the least. At the beginning of an election campaign, and with his chief opponent calling for a substantial tax-cut across the board, it would be asking too much of flesh and blood to stick to an austere programme of economic restraint. Moreover, the package of measures now announced will win some votes. Salary-earners will benefit from the proposed reduction of social security taxes (although the US Treasury will have to borrow from the market to meet the obligations of the years ahead). Business (especially small business) will benefit from the increased depreciation allowances on capital expenditure. But is it realistic to suppose that there are votes in the promise that the budget for basic research should be restored to that first asked for last January, especially when the decision is for now only a promise? On balance, this part of the package appears as disinterested as anything of the kind could be in an election year. Whatever next January may bring, the Administration's protestations must be taken seriously. No doubt a large part of the credit lies with Dr Press.

The question therefore arises as to whether the Administration is going about its intended task in the best way. Protestations and promises, after all, do not make programmes. The chief novelty in the plan for spending the extra £255 million is that the Administration intends deliberately to stimulate industrial research and development. The first thing to say is that the new commitment derives from an idealistic view of the place of research and development as the root of industrial innovation and productivity. (Idealistic, it should be remembered, does not necessarily mean naïve.) Next to the question as to whether it will be possible to vote with a good grace for either of the principal candidates in the coming election, Americans now appear to be mostly preoccupied with the decelerating growth of industrial productivity in the United States. Germany, and even Austria, are becoming more productive faster, and nobody can hold a candle to Japan. The fact that countries such as Britain and Italy are doing even worse than the United States is not a comfort but a warning. But it is also known that the decline of the rate of growth of productivity has been accompanied by a decline of industrial spending on research and development. But is not industrial research the fount from which innovation and productivity spring? And if industry lags, can the government not make good the deficiency by spending public funds on research and development linked with industrial needs?

The calculation is straightforward, familiar and good-hearted. The fact that governments in most other industrialized nations have, in the past thirty years, come to the same conclusions, turned them into policies and then been disappointed in the results does not necessarily imply that the new American initiative will fail. The framework of research is more vigorous and more flexible than elsewhere, and in any case there are lessons to be learned from overseas. No doubt the Administration (if it gets the



chance) will avoid the trap of thinking that research and development will enable inherently uneconomic industries to survive. At this stage, the most important need is that the attempt to reinvigorate American industry by research and development should be openly regarded as an experiment, not as a sure-fire remedy. American governments have some experience of the support of industry by directly financed research programmes, but the conspicuous successes are in fields such as agriculture. The most serious danger is that, presented with the opportunity to help shape the pattern of public support for industrial research and development, Congress will fail to resist the temptation to play pork-barrel politics with a programme that deserves a fair chance.

The record of Congress in the past six months has, after all, been no better than that of the Administration. Chopping and changing is bad for all public programmes where continuity is essential, and especially for research and development. By amending its budget for the coming financial year almost as frequently as the British budget was amended by Mr Denis Healey in his heyday as Chancellor of the Exchequer (between 1974 and 1976), the Administration has inevitably damaged confidence and continuity among researchers. Congress has a right, even a duty, to scrutinize the way in which public funds are spent — that is a large part of its constitutional role. On technical matters, however, Congress is inclined to make judgements of budget proposals on the basis of their declared objectives. Feasibility matters less. This year, no doubt because it is an election year, Congress has been unusually trying. One result is that the budget for the coming financial year beginning in a few weeks is still far from settled. Is it not time that the US government as a whole found a less chancy (and costly) way of deciding how funds should be spent on research and development? As things are, there can be no certainty that the brave schemes the Administration has been hatching will be given a fair trial.

Scares about genetic manipulation

One of the hopes, perhaps unrealistic, of this new decade was that the arguments about the hazards of this or that new scientific development would be couched in more moderate language than used to be the fashion in the 1970s, when Dr George Wald was one of those given to drawing analogies between would-be genetic manipulators and Hitler's would-be eugenicists. And it is true that, for the most part, the argument about genetic manipulation has taken a turn for the rational, partly because experience has shown that the hazards are less serious than some had fairly thought but also because it has become clear that many of the problems thrown up by the practice of genetic manipulation are different in kind from those originally foreseen — problems occasioned by commercial links between academic scientists and industrial companies, for example (see *Nature* 24 July). Yet, it appears, the old Adam lives on, as can be told from a statement put out last week by the Peoples (*sic*) Business Commission, a pressure group in the Nader mould and based in Washington.

The latest declaration from 1346 Connecticut Avenue is, however, such a travesty of the truth that it deserves to be even more widely known than to those on the commission's mailing list. On Wednesday last week, the *New York Times* carried an account of how Dr Francis H. Ruddle and his colleagues at Yale University have been able to incorporate adenovirus and SV-40 genes into the DNA of mouse embryos. The newspaper quite properly quoted the investigators as saying that they had been able to demonstrate the presence of viral genes in somatic cells of mice developed from the manipulated embryos, but emphasizing that they knew nothing as yet of the physiological function (if any) of the genes concerned. They also pointed out that they have not been able to check whether the exogenous genes survive from one generation to the next, but that they hope to do so when their mice are sexually mature. The occasion for the appearance of this newspaper report was the imminence of a meeting in Berlin at which the work will be described; it is said to be due for

publication in the journal *Cell*. That the schemes themselves are adventurous, nobody will dispute. Apart from the restoration of the March cuts of the January budget, which will mean that agencies such as the National Science Foundation and the National Institutes of Health will have more to spend on research grants, there are plans to re-equip university laboratories, to set up "generic technology centres" in partnership with industry and to foster relationships between industry and universities. Sensibly, there are to be consultations between the Administration and the interested parties — universities, industry, and professional and scientific societies — before final decisions are made. In other words, the Administration seems anxious to avoid the trap of imposing a theoretical framework of its own ideas on the pattern of its new spending. That is a sign of grace but also one of the best ways of avoiding obvious pitfalls.

In the long run, however, success will also be determined by broader considerations, many of which have been overlooked in the design of Mr Carter's economic recovery package and the subsequent discussion of it. Whatever may be the merits of the individual components in the package, there is no doubt that the overall effect will be to increase the government's deficit by between \$30 billion and \$50 billion a year. New expenditure, as on research and development, is tiny compared with the cost of the proposed tax concessions and depreciation allowances. Paradoxically, all this has come about when figures suggest that money supply is increasing again, and rapidly. Neither Keynesians nor monetarists would think this a time when the American economy needs further stimulation by deficit financing. Indeed, the consequence of the economic recovery package (if enacted) may be more inflation and more of the damaging consequences for innovation and investment which have become apparent in the past few years. In short, the underlying problems of American industry may be the problems now unhappily familiar elsewhere.

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Plainly, last Wednesday was a black day at the Peoples Business Commission. The statement issued later in the week says that the report "marks the beginning of a eugenics program for America". Messrs Jeremy Rifkin and Dan Smith, described as directors of the commission, say that the development represents "the greatest potential technological threat to the sanctity of life since the beginning of human history". The statement goes on to say that "a few people and institutions now have it within their power to irreversibly alter the biological structure of millions of other men and women and their descendants for all time".

Many will say that foolishness of such a plainly exaggerated form is best ignored. That is too tolerant a view. For small though its direct influence may be, the Peoples Business Commission is well placed to help keep the pot of anxiety about genetic manipulation boiling unnecessarily. It should therefore be widely known that the commission's statement is, put simply, a misstatement of the truth, born possibly of ignorance. For Rifkin and Smith, diligent readers of the *New York Times* though they may be, do not read the scientific literature as carefully. For then they would know that the phenomenon in which endogenous virus genes are naturally incorporated into mammalian genomes is now familiar, and biologically important; that techniques similar to those attributed to Ruddle *et al.* have previously been used with other viruses (and not always in the United States); and that the investigation of this phenomenon may be of the utmost importance in the understanding of diseases such as leukaemia. No doubt the time will come when the *New York Times* reports the incorporation of a human gene into a human genome, but even when that happens it will be hard to justify such an intemperate statement as that put out last week. Is it not high time that the commission's sponsors, whatever their sympathies, found a more level-headed vehicle for prosecuting the case against genetic manipulation?

NSF reorganizes for industrial research

Washington

The great White House hope that scientific research can be harnessed to the cause of industrial innovation has evoked a quick response elsewhere in the United States government. Other agencies are understandably keen to share in the action. At the National Science Foundation, the most immediate result will be to precipitate the reorganisation over which the National Science Board has been agonizing for several months.

Reorganization will dominate the agenda for a public meeting between the acting director of the Foundation, Dr Donald N. Langenberg, some members of the National Science Board and the chairmen of the foundation's advisory committees which has been arranged for Saturday this week (13 September). The meeting will be open to members of the public, who will be able to chip in.

The National Science Board is hoping to have completed these and other consultations in time to make a final decision at its meeting in October. Failure to do so would mean that the proposed reorganization could not be built into the federal budget cycle, which might mean that some changes could not be carried out until October 1982.

The most obvious effect of the proposed reorganization will be to rename the Directorate of Engineering and Applied Science the Directorate of Engineering. More than nomenclature is involved, however — it is intended that each of the existing directorates of the NSF should be more directly concerned with the needs of applied science, while the new Engineering Directorate should vigorously support engineering research in universities.

Novel and explicit mechanisms for fostering industrial innovation are thus not a part of the proposed reorganization. Rather, the NSF is relying on the authority it already has to support industrial research. The scheme for funding joint industry-university projects is doing well. The NSF is also proud of the cooperative research centre in polymer science established at Massachusetts Institute of Technology (and now entirely supported by industrial companies). The analogous research centre for the furniture industry in North Carolina has been less successful.

These proposals are likely to win support. There will be more dispute about the proposal, part of the same package, to set up a Social Science Directorate even though the chief effect would be a repackaging of existing divisions (including the new programme in "decision and management" science). One objection is almost certain to be that "neural sciences", linked with "behavioural science", should not be separated from the rest of biology.

Under the new scheme, the foundation's existing rag-bag directorate (for Scientific, Technological and International Affairs) would take over responsibility for programmes such as those for supporting small business research and affirmative action of various kinds.

By Saturday's meeting, NSF will no doubt have been spurred on by Congressman George Brown's congressional subcommittee, to which Dr Lewis Branscombe (Chairman of the National Science Board) and Dr Langenberg are due to give evidence. Mr Brown is using his bill to set up a National Technology Foundation (unlikely ever to become law) as a way of urging on

everybody in sight the importance of research as a spur to industrial innovation and production.

Meanwhile, the Department of Commerce has stolen a march on other potential competitors for the new funds that may become available in January by announcing its plans to set up three cooperative industrial research centres dealing with welding, lubrication and powdered metal processing. The Department of Commerce will spend \$5.2 million of its own money on the projects, for which proposals are being sought, and hopes that its own contributions to the cost of the centres will shrink to 20 per cent after five years.

Appointments and elections ahead

Washington

After several nail-biting weeks, it now seems that the US Senate will confirm the nomination of Dr John B. Slaughter as Director of the National Science Foundation some time during the week beginning 15 September. It is now thought that the differences, political and procedural, which have arisen over Dr Slaughter's nomination as NSF director (originally announced early in July), will be found to have melted away (or to have been buried) at a meeting of the Senate Committee on Labor and Human Resources due to be held tomorrow, 12 September.

The first stumbling block to Dr Slaughter's confirmation was ironically the reinvocation by Republicans in the Senate of a principle first mentioned by Democrats in the closing months of the first Nixon Administration in 1972 — the doctrine that fixed-term presidential appointments should not be confirmed as soon as this before a presidential election.

Formally, the directorship of the NSF is a six-year appointment, although the statutes allow the President to remove a director from office at any time. In practice, however, a president following such a course would have to contend with the scientific community's view that the directorship of the NSF is not a political appointment in the ordinary sense. Dr Slaughter, when his appointment is confirmed, will be especially hard to dislodge, for he would be the first black American to head an important agency.

Technically, Dr Slaughter's confirmation as NSF director awaits only the vote of the US Senate as a whole, having been recommended ("reported out") by Senator Edward Kennedy's subcommittee on Health and Scientific Research. In practice, the recommendation is flawed by the way in which Democratic members of the subcommittee carried it without a proper quorum. Nobody wants the issue to

be debated by the Senate as a whole. The emollient now discovered is a full meeting of the Committee on Labor and Human Relations at which Dr Slaughter's nomination will be on the agenda but (it is hoped) not discussed. The White House has no doubt been reminded of the quip, usually an ethnic slur, "with friends like that, who needs enemies?"

Dr Slaughter's problems (and the NSF's) may melt away, but Dr Al Carnesale, the putative chairman of the Nuclear Regulatory Commission, is certain to have a rougher ride. Carnesale's nomination, the confirmation of which is in the gift of a subcommittee of the Senate Committee on Environment and Public Works, is frankly acknowledged to be political, and for a specific reason. Carnesale, now a tenured professor at the Kennedy Institute at Harvard University, is committed to the view that breeder reactors are economically irrelevant while the price of uranium is as low as at present. Mr Ronald Reagan, the Republican presidential candidate, disagrees. The chances are that in the circumstances Dr Carnesale will have to bide his time until after the election.

Speculation about other post-election changes is already rife, and the odds are shortening on the proposition that Dr Frank Press, now the President's Science Advisor and Director of the Office of Science and Technology Policy, will finish up next spring as president of the National Academy of Sciences. A spokesman of the NAS confirmed last week that Dr Press is a "leading candidate" to succeed Dr Philip Handler, whose second six-year term ends next June.

Procedurally, however, there is a long way to go before this appointment will be decided. Proposals by the nominating committee have to be approved by the Council of the Academy in October.

The by-laws of the academy require that the nominating committee should "assure itself that each proposed nominee will be

willing and able to serve if elected", which presumably implies a detailed discussion of the terms on which a "proposed nominee" would take the job.

Thereafter the council of the academy will inform the members, who have until 1 December to make alternative nominations (which must be supported by at least fifty members' signatures). If necessary, ballot papers are distributed by 15 December and must be returned by 15 January 1981. In principle, the council can put more than one name to the membership, but it has not in recent years done so. The present procedure, devised only in the 1960s, appears in the 1980s to be calculated to maximize the embarrassment of the "proposed nominee" and of the academy as a whole.

Bell Research Labs

Split possible?

Washington

There is much consternation at the Bell Telephone Laboratories at the unexpected announcement last week that the parent company, American Telephone and Telegraph (AT&T), is to split into two quite separate operating companies. This move, which has been prompted by a decision of the Federal Communications Commission (FCC) made public in May, may be followed at some later stage by a decision that Bell Labs itself should be split in two.

Scientists working at Bell Labs fear that such a development would impair the quality of the organization's research. For the time being, however, uncertainty rather than anxiety rules. No decision has been made as yet about the consequences of the split in the parent company for the research organization.

The crucial decision of the FCC (known as the Final Decision on the Second Computer Inquiry) is the latest stage in the FCC's decade-long attempt to adjust the rules for regulating the public telephone networks to meet new technical needs. Since 1968, the FCC has held that companies such as AT&T should not enjoy the exclusive right to supply terminal equipment for their telephone lines and other channels of communication.

More recently, the difficulties of regulating the common carrier networks have increased with the development of data processing networks, domestic satellite communications and the prospect of other technical developments. For the past four years, the FCC has been searching for a definition that would allow the networks to supply some kinds of equipment but prohibit them from supplying other items.

In its latest ruling, the commission has abandoned this exercise in semantics and opted for a more radical solution. In future, it says, large common carriers of electronic signals must be concerned only

with the development and management of the network. If they wish to supply terminal equipment as well, they must do so through a separately constituted company with a distinctive board of directors, separate premises and so on.

Although the same rule would apply in principle to all telephone companies in the United States, for the time being the FCC has ruled that it should apply only to AT&T and to General Telephone and Electronics, which operates some 8 per cent of the American telephone network in locations scattered from Florida to California.

The response of AT&T to this radical proposal has been equally surprising. Although the company argued strongly against separation in its evidence to the FCC, on the grounds that vertical integration is beneficial, it announced last week that it would go ahead with the formation of two separate companies to be responsible respectively for the operation of the telephone network and the supply of terminal equipment (telephones included).

It is not yet clear whether the separation of the two parts of AT&T will be as complete as the FCC requires. Moreover, the move does not imply that AT&T accepts the commission's ruling — indeed, AT&T is one of thirty objectors who are threatening to carry their case to the courts if the decision is not modified.

The complication for Bell Labs is that the research organization is a jointly owned subsidiary of AT&T and of Western Electric, itself the subsidiary of AT&T responsible for manufacturing terminal and network equipment. The FCC ruling is vague on the implications of the proposed split in AT&T for research and development, saying only that affiliated companies would be required in future to buy research and development services from other affiliated companies at a fair valuation.

One obvious development, that most feared at Bell Labs, is that AT&T may in the end be required to divide both Western Electric and Bell Labs itself into parts separately responsible for communications networks and for terminal equipment. AT&T is also alarmed at the prospect that even separation of activities on which it has now embarked will impede the free flow of ideas between operations and research and development.

If there were to be a split of Bell Labs, the chances are that the largest part would go to the network side. Of the 1980 budget of \$1,255 million, more than 45 per cent was exclusively concerned with network operations. The remainder may be considered relevant to both sides of the telecommunications business.

How soon the future of AT&T and of Bell Labs will be decided is a matter for conjecture. The FCC has set a deadline for the complete separation of AT&T by March 1982, but will now be required to comment on the objections to its ruling, most probably in October or soon afterwards. If the courts accept that the

objectors to the ruling have a case, several further months may elapse before the regulations have the force of law, in which case the deadline would no doubt be put back. Excessive delay is likely, however, to breathe life into the bills now languishing in Congress that aim more directly to regulate the public communications networks.

NPT review conference

No declaration

The Second Review Conference of the Non-Proliferation Treaty ended on Sunday (two days later than originally planned) with a smack in the face for the three sponsoring nuclear powers, the Soviet Union, the United Kingdom and the United States. Although the nuclear powers had been expecting trouble at the conference, largely on the grounds that they had been less diligent than required by the treaty in the pursuit of strategic disarmament and the fostering of civil nuclear developments elsewhere in the world, they were apparently taken aback also to find themselves criticized on the grounds that either Israel (not a signatory of the treaty) or West Germany (which is) had been helping with the development of nuclear weapons in South Africa.

The most immediate cause of trouble was the failure of the conference to agree on a report of the past month's proceedings at Geneva. The drafting process came to a fruitless end in the early hours of Sunday morning, and the conference was concluded later in its day.

By all accounts, the review conference has been rough going ever since the opening speeches were made in public during the first ten days. The government of Mexico appears to have taken the lead in asking that the nuclear powers should agree to a package of demands on arms control, including a declaration that they would abide by Salt II while awaiting ratification of that treaty, impose on themselves a moratorium on nuclear testing and allow the negotiations on the Comprehensive Test-Ban Treaty (conducted up to now between the three nuclear power signatories of the treaty) to be carried on with the UN Committee on Disarmament. For the nuclear powers, the sticking-point last weekend was this demand that such a declaration should be included in the final report of the review conference.

The overriding question now is whether the failure to produce an agreed report will be followed by mass defections from the treaty. The closing speeches at Geneva, including that from the Iraqi chairman, were variations on the theme that nuclear proliferation remains a serious danger, and that the conference had served to draw attention to many of the beneficial aspects of the treaty.

The discovery by several of the

signatories that the quinquennial review conferences offer a means of twisting the arms of the nuclear powers on arms control may paradoxically help to keep some states loyal to the treaty. Others, however, will no doubt be able to use the failure to produce an agreed report as an excuse for withdrawing from the treaty, even though those tempted to take such a step will no doubt be impelled in that direction by separate political considerations.

In the wake of the Geneva conference, the meeting of the Committee on Assurances of Supply of the International Atomic Energy Agency, planned for the end of this month at Vienna, assumes greater importance. It is thought unlikely that the nuclear powers will be spurred to further negotiations on strategic disarmament at least until after the American presidential election.

Plutonium

Dounreay loss

Only a few weeks before the British government is due to make an announcement on the future of the fast breeder reactor, the BBC television programme *Panorama* alleged on 8 September that a total of 35 gram of plutonium from the prototype fast reactor at Dounreay was lost in 1973 and 1976. The United Kingdom Atomic Energy Authority admits that the plutonium has never been accounted for, but says that it could not have left the fuel cycle and was most probably reprocessed or even dumped in a waste repository.

The first case of missing plutonium occurred in 1973 when a routine accountancy check revealed that 10 gram of the element was missing from a can containing the remnants of several spent fuel pins. The pins, originally about 0.2 inches in diameter and 2 feet long, had been irradiated in the reactor core for one year and then chopped up for analysis by chemists and metallurgists. The shortfall in plutonium was equivalent to one fuel pin.

The second case concerned a fuel pin which had been irradiated in the reactor core for one day in 1966. It had then been treated in the same way as the first pin and the can containing it had been stored in a cooling pond. It contained 25 gram of plutonium. In 1977, the can that was thought to contain the remains of the fuel pin was found to contain another type of spent fuel.

The UKAEA says that it investigated the losses and reported the second to Euratom, of which Britain was then a member. A driver of a mechanical digger was hired to uncover all the material in a dump of low-activity waste in the hope that the second lot of missing plutonium would be discovered. The contents of the dump were analysed, but the missing spent fuel was not found.

The UKAEA says that each case was investigated thoroughly, and that the to

missing irradiated fuel pins must either have been reprocessed or dumped in a depository for highly radioactive wastes, examination of which would be too hazardous to undertake. It says the plutonium could not have been removed from the fuel cycle. Its conclusion is that there must have been an error in the system of keeping records of the movement of plutonium. Since the incidents, the UKAEA says, it has tightened up on its procedures for monitoring the movement of radioactive materials around the plant.

The *Panorama* programme was also critical of operations at Dounreay on health and safety grounds. It claims that the driver of the mechanical digger, a deaf-mute, was not properly aware of the hazards of what he was doing and was not given adequate protective clothing. It also cites another incident in 1979 when eight men were exposed to plutonium after handling radioactive waste. None of them was aware that the waste contained plutonium and at the end of their shift it was discovered that they had been exposed to radiation.

About this incident, the UKAEA says that the waste handled by the men contained only one gram of plutonium and that none of the men was later found to have significant amounts of plutonium in his blood or urine. The incident had not been reported because the level of alpha radiation in the air in which the men were working was well below the level at which notification to the authorities is obligatory. An attempt was made, says the UKAEA, to inform the deaf-mute driver of the hazards of what he was doing. He was told to stay in his cab and air samples were monitored for radiation. The levels found were within the safety limits.

The uncovering of the incidents has provoked a strong reaction from Mr David Steel, leader of the Liberal Party, who has called for a ministerial statement on the affair. Even before the showing of the film, the AEA had issued a statement that *Panorama* had exaggerated the significance of these incidents, and had also charged the BBC with having declared an interest in making a film about this achievements

Judy Redfearn

Academic freedom

Talking shops

The Hague

Three papers by Dr Andrei Sakharov, written since his exile to Gor'kii, *Ekspperimental'noi i Teoreticheskoi Fiziki* (*ZhETF*), the most prestigious physics journal in the Soviet Union. This remarkable development was announced by Nobel Laureate Philip Anderson to last week's Conference of the International Sakharov Tribunal of Conscience and Peace.

According to Professor Anderson, the

Editor of the *ZhETF* had initially accepted the papers, which deal respectively with time-reversal, quark/gluon interaction and mass formulae for muons and baryons. At a late stage, however, the censors stepped in to stop publication. The matter, said Professor Anderson, was finally "adjudicated" by the Central Committee of the Politbureau, who decided that publication could go ahead.

The story which amplified by Professor Edward Lozansky, Chairman of the International Sakharov Committee, New York, and a former member of the Moscow Sunday Seminar for Jewish "refusenik" scientists. He told the conference how he had brought the three Sakharov papers to the meeting of the American Physical Society (APS) in April, which decided that if the papers were turned down for political reasons by the Soviet journals the APS would be delighted to consider them. This, thought Lozansky, had probably swung the balance in Sakharov's favour.

Ironically, this revelation of how much Western academic pressure can achieve on behalf of a harassed Soviet colleague was announced at a conference which, in the opinion of several campaigners for academic freedom, should not be taking place at all. One of these is Mrs Tamara Yankievich, Sakharov's stepdaughter-in-law, now resident in the United States. The main grounds for opposing the conference have been the veiled hints from the Soviet side that vociferous action might jeopardize a plan to allow Sakharov quietly to return to Moscow. "Six months after being exiled" was the first date promised for his return; this has now however become "six months after the end of the Olympics".

Unlike the various other "Sakharov" human rights conferences in the past few years, the Hague conference was a human rights conference specifically focused on Sakharov's own plight. As several speakers pointed out, this is a relatively mild plight. For example, Sakharov still has access to a scientific library just across the street from his Gor'kii apartment. Because all the scientific institutes in Gor'kii are involved in classified work, however, there is no possibility of his having any contact with fellow scientists in his place of banishment.

In his presentation to the conference, Professor John Ziman of the University of Bristol suggested that one of the reasons scientists are particularly concerned with Sakharov's case is that his involvement with problems of academic freedom and human rights generally grew out of his disillusionment with a career in nuclear weaponry — what Ziman called "black science" — like black magic, "the application of knowledge in the power of evil".

His "pilgrimage", as Ziman called it, is therefore of particular concern to all scientists, "hard put to it these days to defend our traditional norms — the universality, the disinterestedness, the openness of a transnational community bringing beneficial knowledge to all".

Coincidentally with the Hague conference, two other meetings concerned with the political repression of scientists also took place last week. In Amsterdam, there was the annual meeting of the International Council of Scientific Unions (ICSU) and of its specialist committees, including the Committees on the Free Circulation of Scientists and Safeguards of the Pursuit of Science. This committee, which compiles a register of cases of scientists subjected to unwarranted restriction, is less well known than it should be. During the past year, only some seventeen such cases were added to the register.

The particular roles of scientific and learned societies were stressed at the other

meeting last week — the half-day session on scholarly freedom and human rights at the Salford meeting of the British Association.

At such conferences, the question of how to translate concern into concrete action is usually answered only vaguely. On this occasion, Dr Louis Cohen, secretary of the Physical Society (London), put forward a fourteen-point scheme of progressively more severe responses that learned societies might make (see below).

By way of demonstrating what may be achieved by pressure from professional colleagues, Professor John Charap of Queen Mary College, London, was able to announce at the BA meeting an unprecedented success in the case of Yurii Gol'fand, his fellow researcher on supersymmetry. Gol'fand, who was dismissed some years ago from his post as senior researcher at the Lebedev Institute of Physics in Moscow following his application to emigrate to Israel, was, a few weeks ago, reinstated.

Vera Rich

Marks of reproof

What follows is a summary of Dr Leslie Cohen's suggestions as to how learned societies may help colleagues elsewhere whose academic freedom or human rights are restricted (see text).

1. Statement by the President of the Society.
2. Formal protest by Officers of the Society (published in the Society's bulletin).
3. Provision of facilities for protest meetings, press conferences.
4. Official letter of protest to head of State Party/Academy.
5. Dissemination of information on individual cases to members.
6. Advising members to refuse to
 - (a) attend meetings in offending country;
 - (b) invite scientists from offending country to conferences;
 - (c) allow visits of scientists from offending country, to scientific establishments/laboratories;
 (Note in special cases, visits may be made contingent on discussion of human rights issue in question).
7. Organization of petitions by members on specific case.
8. Granting of recognition and/or publicity to "alternative" meetings and seminars organized by banned scientists.
9. Organizing and/or supporting meetings relating to the work of one particular scientist suffering repression.
10. Sending delegation to investigate cases and intervene if possible.
11. Providing journals free to banned scientists.
12. Providing financial support to exiled scientists.
13. Severing formal relations with academies/societies in offending countries.
14. Causing expulsion of academies/societies of offending country from international organizations.

Hoyle on life

No dissent

It would be hard to imagine an event more like that of Daniel entering the lion's den than last week when Sir Fred Hoyle, brandishing his theory that influenza epidemics are caused by extraterrestrial viral material, gave a seminar at the National Institute for Biological Standards and Controls in Hampstead, London. In the event, Sir Fred escaped almost as unscathed as Daniel, although perhaps for different reasons.

Hoyle has evolved his theory in conjunction with Professor N.C. Wickramasinghe of University College, Cardiff, with whom he has recently collaborated on a series of papers on the occurrence of biochemicals in interstellar space. The theory that viruses might also be present in space is a natural extension of that line of work. It is worth postulating, according to Hoyle, because of some anomalies in the epidemiology of influenza which defy the conventional explanation that the virus is passed from person to person, with animals acting as a reservoir.

The anomalies to which Hoyle points are few but striking. He cites, for example, outbreaks of influenza in boarding schools where the great majority of cases occur simultaneously quite unlike the expected, progressive spread from one or a few initial cases. More strikingly he points to reports of the simultaneous outbreak of influenza in the USA and India during the 1918 pandemic. What better explanation for such phenomena than that a space-borne factor descends to trigger the influenza in all cases at the same time? And, in support of that notion, Hoyle marshals plenty of evidence of atmospheric events that could account for the odd incidence of influenza.

A version of Hoyle and Wickramasinghe's theory that has been circulating in preprint form proposed that individuals carry as part of their own genes the genetic information for each of the many types of influenza virus and that there was a separate space-derived, activating factor for each.

Now, however, he is persuaded that this version of the theory is inadequate in part because of overlooked evidence and in part because biological systems have a good deal more slack than he, as a physicist, had been aware of. As a result the theory has become more complex and now involves a coincidence of three virus-related components, two of them space-derived, for infection to occur.

In becoming so complex, the credibility of the theory has undoubtedly been stretched more than ever. There is also the question of whether or not the basis of the theory — the anomalous epidemiology — is sound. Thus in the discussion that followed Hoyle's talk, Dr A. J. Smith of the Influenza Research Unit at St Luke's Hospital, Guildford, outlined his unit's study of the spread of influenza in a school. Unlike the evidence cited by Hoyle, Smith's data fitted the classical progressive spread of the disease, from a single person, to all those who finally succumbed.

That, however, was the only criticism of substance put to Hoyle in the discussion period. One respected influenza expert was prepared to venture the view in private that whereas he and others had no time for Hoyle's theory they were too polite to say so; "in the USA he would have been eaten alive".

And so it came to pass that, for reasons perhaps more British than biblical, Sir Fred survived the tame lions of Hampstead.

Peter Newmark

Electric vehicles

UK content

Government and industrial support for British research and development on electric vehicles should continue at its present level even though it is likely to be many years before electric vehicles become commercially viable. This is the main recommendation of the first report of the House of Lords Select Committee on Science and Technology, published last week.

The technical problems of developing advanced batteries suitable for use in private cars and of building an infrastructure for overnight recharging are identified as the main obstacles to the early success of the all-electric car. Another problem is that most of the advantages accrue to society and the electricity generating industry and most of their disadvantages fall on their owners. Their commercial success is therefore unlikely much before the petrol-run car becomes exceedingly expensive to run, probably towards the end of the century.

The Lords committee warns that there is a danger of Britain becoming complacent about the need to develop alternatives to the internal combustion engine because the availability of North Sea oil might make petrol-run cars economic in Britain after they have ceased to be so in other countries. The committee recommends that the government encourage electric vehicle manufacturers to continue their research at least at the present level, which it finds satisfactory, by continuing to give financial support matched in some way to the manufacturers' own investment. Government should also be prepared to make product-launch money available "to get over the volume-production lump in the next few years". The committee does not, however, suggest that electric vehicle research be centrally planned, finding the present system of manufacturers applying for grants on an *ad hoc* basis satisfactory. It has seen no evidence of any overlap of research.

There is one area, however, in which the Lords say that more work could be done. Hybrid vehicles, propelled by a combination of battery and internal combustion engine, are being vigorously explored in Japan and the USA, but have so far received little attention in Britain. They offer the most promising prospects for private use by overcoming the pure electric car problem of short range, typically 50-70 miles. They also do not necessarily depend on the development of new advanced batteries. A major disadvantage, leading to high capital cost, is that incorporating two different propulsion systems in one vehicle is technically very difficult. Nevertheless, the government should be prepared to make a "small" amount of public money available for testing them, says the committee.

The future of electric vehicle development lies with large companies prepared and able to invest heavily in research and development and not with small businesses set up to build one or two specialized vehicles, says the report. So far, the manufacturers of batteries and components, such as Lucas and Chloride, have invested much more than the major car manufacturers. A possible reason, put to the committee by BL Technology, a subsidiary of British Leyland, is that vehicle manufacturers add only 30 per cent of the final value of an electric vehicle, the electrical component manufacturers standing to win the biggest reward.

The committee hopes that the car manufacturers will eventually be enticed to invest in electric vehicle development by the prospect of major markets in countries which, unlike Britain, cannot rely on a steady supply of oil into the 1990s. It would be a pity, it says, if Britain were to lose its lead in electric vehicle technology now, only to be forced later into buying from abroad because it had been lulled into a false sense of security in the 1980s.

Judy Redfearn

US petroleum

Using less

Washington

Officials at the Department of Energy appear to share with the superstitious the view that to talk about good luck is to ensure that it will disappear — "don't count your blessings" and all that. Accordingly, not much is being made of the dramatic reduction of crude oil imports to the United States in the past year, which in the first eight months are down by 17.2 per cent between 1979 and 1980.

According to the Department of Energy's *Weekly Petroleum Status Report* for 29 August, imports of crude oil and refined products averaged 6,907 million barrels a day in the first 234 days of 1980, compared with a total of 8,341 million barrels a day in 1979. If this keeps up, President Carter's promises at the Venice summit on oil imports will be amply fulfilled.

The diffidence of the statisticians responsible for the weekly statistics is partly technical. The weekly *Status Report*, although admirably up to date, is based on returns from oil companies and crude oil refiners, and is therefore not a direct measure of actual consumption. Perhaps, it is said, the reduction of imports reflects reduced inventories in the distribution network. The rapid attrition of gasoline service stations certainly implies that many retailers' gasoline tanks have been emptied for the last time. The statisticians appear not to dare to take heart from the

supposition that most retailers going out of business are doing so because they are selling less.

The statisticians are also diffident because they do not understand why the most recent round of price increases for petroleum products should have demonstrated, after a decade of apparent price inelasticity, that even Americans will buy less gasoline and fuel oil if the price goes up sufficiently. Could it even be that some non-quantifiable consideration, such as the continued presence of American hostages in Iran, has changed people's buying habits?

Whatever the truth, the facts are clear. Between the first eight months of 1979 and the corresponding period this year, gasoline production for the US market has declined by 6.8 per cent, to an average of 6.6 million barrels a day (still more than a third of total volumetric production of 17.6 million barrels a day of all oil products). Distillate fuel oil is also down by 14.2 per cent, partly because householders and commercial consumers have turned down their thermostats, partly because oil companies are chafing at state and even more local price restrictions. Already there are fears that if the coming winter is cold, it may also be hard.

During the same period, stocks of crude oil and of petroleum products have soared. Primary commercial stocks have increased by more than ten per cent in the past year. Gasoline is in especially good supply. Fuel oil is, however, short.

The most obvious disappointment in the statistics so far available for 1980 is that US oil production has hardly changed since

Saturn seen in Voyager sights

The accompanying photograph, released at the end of August from the Jet Propulsion Laboratory in California, is one of the first to have been gathered from the spacecraft Voyager 1, now on its way from Jupiter to Saturn. Between 22 August, when the first photographs were obtained, and 24 October, visual observation from Voyager 1 will consist entirely of single-frame photographs. Thereafter, the narrow-angle camera on the spacecraft will be used to collect mosaic photographs of the visible surface of Saturn and of the rings and to search for satellites at present unknown.

The closest approach to Saturn will occur on 12 November, when the spacecraft will also come closest to the satellites Tethys, Mimas and Enceladus. The closest approach to Titan, one of the principal targets for the cameras, will occur on 11 November. Visual observation of Saturn will continue at least until 18 November.

Visual observations are, of course, merely the most memorable of the observations made by planetary spacecraft. Voyager 1 is also equipped with magnetometers, infrared sensors (which will, in particular, produce a map of the surface of Saturn) and devices for recording the interplanetary particle flux and for measuring the size and density of material particles in the rings of Saturn.



Saturn from Voyager 1 on 24 August, 1980. The dark band on the equator is the shadow of this rings. The three bright specks are satellites.

1979, increasing by only 2 per cent. One explanation of this curious price insensitivity is that potential oil producers are waiting until the price of "new" oil is decontrolled towards the second half of 1981.

The economic consequences of this changing pattern of consumption are plain to see. The three major automobile manufacturers are in the throes of launching their newest small cars — Ford unveiled its new Escort last week — with apprehension and despondency. If the cars do not sell, consumers will buy imported cars and the auto industry will collapse still further. But the profitability of small cars is not nearly as great as that of the old gas-guzzlers.

Using the standard relationship but on the assumption that coal production will continue rapidly to increase, the American Petroleum Institute is now guessing that by 1990 oil consumption in the United States will decline to an average 8-9 million barrels a day, with rather less than half of this imported. Even this estimate may be conservative, for the impending decontrol of petroleum prices may be an unexpected stimulus to drilling. Since the decontrol of natural gas prices during 1979, proven reserves of natural gas have increased more quickly than in the previous decade.

Romanian energy

Partners to-be

Romanian President Nicolae Ceausescu has found the answer to current and future problems of the development, efficient and economic use of raw materials and energy resources, according to the leading Party newspaper *Scinteia*. Reviewing the latest volume in the series "From the Economic Thinking of President Nicolae Ceausescu", *Scinteia* points out that the President was interested in the problem even before the energy crisis. This is, perhaps, not surprising as Romania, once so oil-rich that an oil-rig was depicted on the state coat of arms, has now sunk to the status of a net oil importer.

Judging from the review Ceausescu's book *Energy and Raw Materials Resources* (not yet available in the West) seems to reiterate the draft directives for the 1981-85 five-year plan and the long-term guidelines till 1990, recently accepted by the Joint Plenary Meeting of the Central Committee of the Romanian Communist Party and the Supreme Council of Economic and Social Development.

These guidelines provide for a multilateral energy strategy, to include: the expansion of geological prospecting throughout the country and the Black Sea continental shelf, the speeding up of hydroelectric projects, so that the 30 per cent exploitation of hydroelectric potential of 1980 will reach 45 per cent in 1985, 65 per cent in 1990 and 100 per cent in the year 2000, utilization of low grade and less accessible fuels such as shale, and the expansion of the nuclear energy

programme (660 MW capacity by 1985, 3,960 MW by 1990 and 10,000 MW by 2000).

Considerable emphasis is also to be placed on conservation, recycling and what looks remarkably like energy rationing — described by official policy hand-outs as "fixing rational ceilings on heating and lighting". Finally, Romanian scientists, whose work has been more firmly harnessed to the needs of the economy than in any other socialist country, will take part in wide-ranging interdisciplinary research to capitalize on the country's geothermal, Sun, wind and tidal resources, as well as biomass conversion. According to the directives, by the year 2000 these resources should account for some 20 per cent of Romania's energy production (by then nuclear energy will have reached 17 per cent).

Vera Rich

Rural communities

Living now

What sort of society could the future inhabitants of rural areas find themselves living in? A recent idea for a rural community for the future has come from the Dartington Hall Trust in South Devon, England in the form of a model village built on a scale of 1:250. Recognizing, no doubt, that the future can never be planned by the past, the Dartington model has been designed more as an "exercise in the imagination" than as a blueprint for a real village.

Thinking about the future of rural communities fits well into the work of the Dartington Trust, which was set up in the 1920s with the wealth of Leonard and Dorothy Elmhirst to bring back life to rural communities which has been depleted during the industrialization of the nineteenth and twentieth centuries. Most of Dartington's work consists of encouraging small industries, not necessarily associated with rural life, and cultural and educational activities in the countryside of Devon. Its latest venture, however, is more theoretical. According to Maurice Ash, the Trust's chairman, the model village is an

Dream in balsa wood



attempt to "speak a new language" by thinking differently about the world in terms of qualities rather than quantities.

That is not to say, however, that the "high" technology of the industrialized world has been ignored. The idea, according to Andrew Page, the brains behind the model, is to achieve a marriage of the microelectronics and green (ecological) revolutions. The model is for a community of about 2,000 people which aims to be as self-sufficient as possible by making use of both "low" and "high" technologies, the emphasis being on energy saving and preserving the quality of the environment. Electricity is supplied mainly from hydro and aerogenerators, transport within the village is by bicycle, horse and cart or on foot, as much local produce as possible is grown around the periphery of the village using labour intensive methods and waste recycling is done on site to produce clean water supplies and methane and other biogases. The village medical centre is designed to offer "alternative therapies" as well as modern medicine.

High-technology services come into their own when the village has to communicate with the rest of the world. Hence a community transport depot houses cars, lorries and vans for those needing to travel outside the village. But the most significant feature is the "cottage office" which houses a "resource centre" offering not only telephones, telexes, photocopying machines and secretarial and accounting services for local businesses, but also television with teletext, Viewdata and other link-up services for worldwide electronic communication. This acknowledgement of the microelectronics age also means that the village can offer educational facilities for all age groups.

Small-scale industry, for producing goods for sale outside the community as well as within it, is based on a mixture of high and low technologies. Few inhabitants would have only one job, the idea being to create job fulfilment by blurring the distinction between work and leisure and engaging people in different tasks at different times. Most people, for example, would spend quite a bit of time farming the green belt surrounding the village or digging their allotments, in addition to their main job. One of the main criteria for using high, labour-saving technology would be the elimination of boring and repetitive tasks.

It is unlikely that the model village will ever become a reality. But some of the ideas and detailed design work that have gone into creating it may be useful in less ambitious projects to build real futuristic societies. The Town and Country Planning Association and Greentown Group, for example, are currently looking for a site to build a small futuristic community of houses and small businesses. And Milton Keynes has already shown an interest in building such a community on a 25 acre site just outside the town.

Judy Redfearn

NEWS AND VIEWS

The elementary Universe

from M.G. Edmunds

SINCE nearly every object in the Universe contains more than one chemical element, it is perhaps not surprising that the experimental determination and theoretical explanation of the abundances of the elements is a rich field of research. The relevance of abundance studies to the whole of astrophysics was very evident in this summer's Santa Cruz Workshop*.

The initial Big-Bang left only isotopes of the very lightest elements, and everything from carbon onwards must have been built up during the evolution of the Universe out of hydrogen inside stars. The synthesis of most elements has been assumed to occur by nuclear reaction chains in the extreme temperatures and densities which occur when a star explodes as a supernova. Although there was much indirect evidence for this basic assumption, it is only recently that direct observational evidence has been found. The analysis of the optical spectra of remnants from such explosions in our own Galaxy (the remnant Cas A) and from a remnant in an irregular galaxy (NGC 4449) by R.P. Kirshner (University of Michigan) does at last provide a clear demonstration that synthesis reactions have occurred. The debris of the explosions can be seen streaming out at some thousands of kilometers per second, and has a composition dominated by freshly-synthesized heavy elements (particularly oxygen) with very little unprocessed hydrogen remaining.

Supernovae divide up into two main classes — Type I and Type II — on the basis of the way their phenomenal light emission fades in the first few months after explosion. The exact nature of the stars which give rise to the two different types remains uncertain, but Type II may originate from massive stars (perhaps larger than twelve solar masses). Type I might originate from single stars of somewhat lower masses, or (as originally suggested by J. Whelan & I. Iben *Astrophys. J.* **186**, 1007; 1973) from a

binary system of stars when matter is transferred from one component of the system to the other, more condensed star. Models of this second kind were particularly popular at Santa Cruz. Both T. Weaver (Livermore Laboratory) and K. Nomoto (Goddard Space Flight Center) investigated a version in which the gradual transfer of helium onto a white dwarf star results in conditions which give a sudden detonation of nuclear reactions. The exponential pattern of the decay of light output from the resulting explosion gives a fairly direct indication of nuclear syntheses, since the energy output originates in the radioactive decay of freshly synthesised ^{56}Ni which decays to ^{56}Co in a few hours, and then to stable ^{56}Fe in a few months. Models presented by T. Axelrod (Livermore Laboratory) suggest that up to one solar mass of decaying nickel may be involved in a typical Type I supernova, and he claimed that spectral lines of ionised cobalt can be seen in the optical spectrum with a strength and time variation consistent with the decay of large amount of ^{56}Co .

Exactly how the supernovae explode, and how much of the parent star is neutronised and left behind as a highly condensed stellar remnant, remains uncertain — although everyone seemed to agree that the amount of rotation present is likely to be very important in determining the dynamics of the explosion. A particularly intriguing possibility is that, if the pre-supernova star sheds enough of its outer layers for non-explosive mass loss, then a nuclear runaway might occur without a violent release of optical radiation (although there would probably be copious X-ray emission). Thus it may be possible to form neutron stars without an obvious optical supernova event, although a recent lowering of the estimate of the birthrate of pulsars in the Galaxy to one every hundred years (M. Arnaud & R. Rothenflug *Astron. & Astrophys.* **87**, 196; 1980) indicates that optical supernovae may, in fact, provide sufficient neutron

stars to account for the observed population.

Supernovae also provide one of the best means of establishing the distance scale to external galaxies, and D. Branch reported work by himself and others at the University of Texas on a supernova in the galaxy M100 which suggests a Hubble constant near $60 \text{ km s}^{-1} \text{ Mpc}^{-1}$. The result was greeted with relief by many participants because some recent higher values had implied an age of the Universe much smaller than that estimated from radioactive isotope ratios and from ages of old star clusters suggested by stellar evolution theory.

The relative contribution of Types I and II supernovae to the overall heavy element abundances in the Universe is also uncertain, (see, for example, B.M. Tinsley *Astrophys. J.* **229**, 1046; 1979). The observation that oxygen seems more abundant relative to iron in very old stars (which are a sample of early Galactic history) than it is in young stars, prompted S. Woosley (Lick Observatory) to propose on the basis of nucleosynthesis calculations by himself and T. Weaver that oxygen is predominantly synthesised in Type II supernovae and that there were relatively more of these massive stars early on. The major source of iron in the Galaxy would then be subsequent Type I supernovae.

On a galactic scale, the existence of abundance gradients in our own and other large spiral galaxies appears to be well established, in the sense that the central regions have more heavy elements per unit mass than do the outer parts. The best indicators so far are the photoionised HII gas regions, whose spectra have been extensively observed by American, British, French and Mexican groups over the past few years. For our own Galaxy, dust extinction in the Galactic plane makes optical observation difficult, but P. Shaver

*The Santa Cruz Workshop in Astronomy and Astrophysics on "The Origin and Distribution of the Elements" was held from July 6 to July 18 at the University of California, Santa Cruz.

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(European Southern Observatory) presented radio recombination-line data which show a definite gradient in the electron temperatures of the gas regions as a function of their position in the Galaxy. This may be directly interpreted as an abundance gradient since the temperatures are largely determined by the cooling of the nebula by radiation from the heavy atoms — if abundances vary, then so will the temperatures. For other galaxies there is some indication that the gradients may be flatter in earlier Hubble types, but this correlation of gradient with morphology is unclear, particularly as M. McCall (University of Texas) presented evidence for very different gradients in two galaxies (M101, NGC 6946) which have been classified as exactly the same morphological type.

Even more problematic was P. Wamman's (Caltech) conclusion that the isotope ratios $^{12}\text{C}/^{13}\text{C}$, $^{16}\text{O}/^{18}\text{O}$, $^{14}\text{N}/^{15}\text{N}$ (as accurately determined from radio molecular line spectra) do not vary significantly across the disk of our own Galaxy, except right in the Galactic Centre. This result is very surprising since the production and destruction rates for the rarer isotope of each of these pairs would be expected to vary considerably if there exists an overall abundance gradient across the Galaxy. Although there may remain some uncertainty in the correction of the molecular line data for fractionation effects in the clouds where the molecules are found, it seems that a radical re-think of the production mechanisms (particularly of ^{18}O , ^{15}N) may be required. Why the Galactic Centre should be different is anyone's guess, but M. Peimbert (University of Mexico), with his discovery of a small Seyfert-like nucleus in M81, reinforced the idea that perhaps all galactic nuclei in spirals are peculiarly active.

The peculiar variation in abundance ratios observed between different stars in some globular star clusters remains something of a mystery. Some of the variations can be ascribed to nuclear processing during the normal evolution of the stars, but variation in other elements seems to force acceptance of the view that there were considerable (and peculiarly selective) inhomogeneities in the initial composition of the gas cloud out of which the clusters formed. But even the anomalies explicable by nuclear processing are not well understood, because according to existing theory many of the stars have not reached a sufficiently advanced evolutionary stage for the products of nuclear reactions in the core of the star to be mixed up by mass motions and reach the surface where they are observed.

The possible inadequacy of mixing theory in stars was stressed by S.E. Persson (Mount Wilson Observatories) for the so-called 'carbon' stars in the Magellanic Clouds, where again there is clear evidence of nuclear processing and mixing at

luminosities well below theoretical predictions. Evolved stars undergoing mixing are also a site for synthesis of elements which are formed by repeatedly capturing neutrons at a rate which will allow each successive product of the reaction to β -decay (if it is unstable) before the addition of another neutron. A rather nice demonstration of the occurrence of this reaction chain under different conditions was given by H.E. Bond (Louisiana State University) in his differentiation between 'classic' and 'marginal' Barium stars. In the 'classic' stars the flux of neutrons in the stellar interior has been high enough to add sufficient neutrons to the iron group elements (atomic number $A \sim 56$) to synthesize considerably heavier elements, giving the characteristic observed over-abundance of Barium ($A = 138$). The 'marginal' stars show evidence of only a small amount of neutron addition, giving over-abundances of Strontium and Yttrium ($A = 88, 89$) but insufficient addition to build up a large excess of Barium. Here is concrete evidence of different amounts of nuclear processing during the normal life of stars.

The study of such systematic variations in abundances is of vital importance in sorting out the theory of mixing, and hence tightening up the (still very real) uncertainties in stellar evolution models.

Typical of the spin-off from abundance studies is the discovery by B. Carney (Carnegie Institution of Washington) that there exist some old stars near the Sun with space velocities very much in excess of the velocity of escape from the Galaxy which would be calculated on the assumption that the Sun is near the edge. The continued retention of such stars in the Galaxy strengthens the case for the existence of a large amount of unseen mass outside the Sun's Galactic radius.

The improvements in mass and charge resolution of cosmic ray experiments over the past few years are now beginning to bear fruit. After the observed fluxes have been corrected for reaction during the propagation from their source to Earth, there are no major differences between cosmic-ray heavy-element composition and that observed in the interstellar medium.

This suggests the origin of Galactic cosmic rays is accelerated interstellar material, rather than freshly-synthesized particles thrown out from the interior regions of a supernova explosion. With present resolutions, one anomaly does seem definite; this is the greater abundance of ^{22}Ne relative to ^{20}Ne in comparison to that observed in most solar system material. Numerous models were invented to explain this, but perhaps the most attractive was the suggestion (S.E. Woosley) that material which had undergone reactions involving the fusion of helium in stars (a product of which would be ^{22}Ne) is preferentially accelerated into cosmic rays. The acceleration would presumably act on this matter after it had been lost from the star.

Both the cosmic-ray and gamma-ray observers were quick to point out the serious dilemma facing them — more statistics are needed, and their next generation detectors will have much superior capability, but the number of flights on which they can launch their equipment into orbit is dwindling rapidly.

Another aspect of this widely-ranging workshop was discussion of the isotopic anomalies found in certain discrete regions (or 'inclusions') in meteorites, the anomalies contrasting with a remarkable uniformity of isotopic composition (apart from some well-understood fractionation effects) in most solar system material. The anomalies may result from large inhomogeneities in the isotopic composition of the early solar system nebula, although H. Norgaard (Niels Bohr Institute) outlined a scheme for synthesising ^{26}Al (which decays into ^{26}Mg , one of the principal anomalous isotopes) in red giant stars and hoped that dust condensing in the giant stars' atmospheres would provide suitable transport to the early solar system for the radioactive aluminium and its decay product. But the anomalies occur in many different elements, for example G. Wasserburg (Caltech) had found a huge 200% anomaly in $^{107}\text{Ag}/^{109}\text{Ag}$. Confusion is added by the lack of some anomalies which would be expected on nuclear production explanations for other isotopes. Although much beautiful experimental work has been done in discovering these anomalies, their origin remains obscure and must rank as one of the major cosmochemical puzzles.

There was, however, at least one area in which good agreement was reached. This was an estimate of the pre-galactic (i.e. Big-Bang produced) helium abundance. Work by several groups (particularly Peimbert and his collaborators) based on the analysis of spectra of HII regions in irregular galaxies, gave a primordial helium mass fraction of $Y = 0.225 \pm 0.015$. This is in excellent agreement with an estimate of $Y = 0.23 \pm 0.01$ which V. Castellani (University of Rome) made for the helium abundance in a particular type of globular cluster which could be rather well constrained on the basis of stellar pulsation and evolution theory. Since the synthesis of helium in the Big-Bang is influenced by the overall density of the Universe and the details of particle physics, G. Steigman (Bartol Research Foundation) gave an impressive argument that this low value (which is significantly lower than the often-adopted $Y = 0.25$) places constraints on particle physics if a density for the Universe can be assumed. In particular, it may limit the allowed number of neutrino types (whose flavours had been threatening to outnumber those available at the local ice-cream parlour) to only two or three.

Many other problems were discussed at Santa Cruz, and if the function of a good workshop is to raise more questions than it answers, then this one certainly succeeded! □

Why are there fewer frogs and lizards in Southeast Asia than in Central America?

from Robert M. May

It has long been suspected that the densities of floor-dwelling amphibians and reptiles are lower in the rainforests of Southeast Asia than in those of Central America.

The first quantitative analysis, however, was made by Scott (*Biotropica* 8, 41; 1976), who compared his data from two Costa Rican forests with that of Lloyd *et al.* (*Am. Nat.* 102, 497; 1968) from Nanga Tekalit in Borneo. Inger (*Am. Nat.* 115, 761; 1980) has recently widened the scope of such comparisons, combining his own additional data from Bornean, Malayan and Thailand forests with Heatwole and Sexton's (*Am. Midl. Nat.* 75, 45; 1966) results for Panamanian rainforests. The main results are summarized in the Table.

By and large, the numbers in the Table are for floor-dwelling creatures, omitting arboreal and riparian-terrestrial animals (though the Panamanian study includes some lizards captured on trees). Inger notes that turtles and snakes were found too rarely for it to matter whether they are included; similarly, the Costa Rican numbers are little affected by adding in salamanders (as Scott did).

Inger presents further data on the daily catch of frogs and lizards in two other rainforest sites in Borneo, and in three sites (rainforest, logged forest, rubber planting) in Malaya. Although absolute estimates of individuals per m² are not available for these sites, the number of catches per day in the rainforest sites were so similar to those in the Old World sites listed in the Table that it is reasonable to assume the densities would also have been similar. On the other hand, the catch per day in the two disturbed sites (logged forest and rubber plantings in Malaya) were more than twice the rates in undisturbed Old World rainforests. Inger suggests this may be due to environmental factors, or to the abundance of species that might be called commensals of man; it could alternatively be due to the enhanced abundance often associated with the 'r-selected' species that characterize disturbed habitats.

The Table shows a consistent pattern, with the total density of frogs and lizards in rainforests in the New World being an order of magnitude higher than those in Southeast Asia. This overall pattern conceals some interesting details. One such is the reversal of the preponderance of frogs over lizards as one goes from Borneo to Thailand; Inger and Colwell (*Ecol. Monogr.* 47, 229; 1977) attribute this to the relatively dry, harsh seasonal climate of the Thailand sites, which would favour reptiles over amphibians.

What is the explanation for the striking

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differences revealed in the Table? In terms of ecological roles, species of frogs and lizards dwelling on the floors of tropical forests are very similar; in both Old and New Worlds, the majority feed on arthropods and other invertebrates, and both groups inhabit the same kind of microhabitat. The total number of species of frogs and lizards (species richness) is not

Comparison of densities of floor-dwelling amphibians and reptiles in lowland (<500m) forests of Old and New World tropics.

Place	Individuals per 100 m ²		
	Frogs	Lizards	Combined
Borneo: Nanga Tekalit	1.31	0.25	1.56
Thailand: Sakaerat			
evergreen forest	0.12	1.03	1.15
deciduous forest	0.27	1.22	1.49
Costa Rica: Ricon de Osa	11.6	3.9	15.5
Costa Rica: La Selva	14.7	2.8	17.5
Panama: Silugandi	29.8	15.4	45.2

significantly different between the two hemispheres. The abundance of snakes or other important predators of frogs and lizards does not show systematic differences.

Scott attributes the difference in density to there being less production of litter in Bornean forests. But Inger shows some of the estimates of litter production in Bornean forests used by Scott are inappropriate (coming from impoverished heath forests), while others have been revised upward by later work. It does not now seem that production of litter varies much among the major tropical rainforests.

Inger's explanation begins with the observation that a high proportion of the

trees in Indo-Malayan rainforests are members of one family, the Dipterocarpaceae. Dipterocarps are noted for their synchronized mast fruiting, which often involves more than 100 species over thousands of square kilometers; the intervals between such reproductive explosions exceed one year. This phenomenon has, however, not been reported for trees in Neotropical forests. Inger suggests that "Mast fruiting should lead to reduction in abundance of an entire class of arthropod primary consumers during non-mast years. In turn, this reduction should affect the total size of the insect population and, therefore, the numbers of insectivorous secondary consumers, such as frogs and lizards. Since ripe dipterocarp seeds do not hang but fall to the ground, it is likely that a reduction in the numbers of seed-eating insects and their arthropod predators would have its greatest effect on the food supply of terrestrial rather than arboreal frogs and lizards."

Inger notes that his hypothesis is susceptible to several tests. If his ideas are correct, both the total number of insects, and the proportion comprising fruit and nut eating insects, should be lower on the floors of Southeast Asian forests than in the Neotropics. Similarly, we would expect to find fewer insectivorous birds feeding on or near the ground.

If Inger's suggestions survive these tests, the problem will have been pushed back one notch. We will then need to understand whether it is historical accident or systematic environmental differences that have produced spectacularly synchronized mast fruiting in Southeast Asian forests and not in the New World. □

Neurotoxicity: Mechanisms explored and exploited

from M.K. Johnson

New knowledge of the mechanism of delayed neurotoxicity of some organophosphorus esters is enabling realistic assessment of hazardous levels of exposure. Tri-aryl and mixed alkyl-aryl phosphate esters are widely used as hydraulic fluids, flame retardants and plasticisers and, in both agriculture and disease vector control programmes, organophosphorus pesticides are now used in preference to the persistent halogenated hydrocarbons. Some, but not all, of these esters cause delayed neuropathic effects when tested in sensitive species such as hens

or cats and similar neuropathies have occurred in man after ingestion of organophosphates. There is an interval of 10-15 days between ingestion of a neuropathic ester and the onset of clinical signs and, until recently, clinical observation has been the most sensitive tool of the neurotoxicity tester.

At a recent conference* the primary causal molecular changes were presented and the ways in which this knowledge could

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be applied in design and safety evaluation were discussed. M.K. Johnson (MRC Toxicology Unit, UK) showed that many macromolecular sites in nervous tissue are phosphorylated within a few hours of dosing hens with a radio-labelled neuro-pathic organophosphate. These sites have been dissected into sets and sub-sets by selective use of inhibitors and the delayed neurotoxicity protein phosphorylation site identified unambiguously in one sub-set. The initiation mechanism comprises a phosphorylation of the target protein followed by a bond-fission to form a charged monosubstituted phosphoryl group bound to this site.

Reports at the Round-Table focussed on the fact that the phosphorylation site can hydrolyse phenyl valerate and that toxic attacks on the site can therefore be assessed using the 'neurotoxic esterase' (NTE) as a biochemical monitor. Even at doses producing no clinical or histological effects during the critical 10-15 day period after dosing a response of NTE inhibition at 1-2 days can be observed and quantified. This provides a means to grade the toxic response in a way superior to the previous yes-or-no evaluations.

M. Lotti (Padua University) has studied autopsy samples of human nervous tissue. He finds that *in vitro* the inhibition characteristics of acetylcholinesterase (AChE) and NTE from man are similar to those from the hen. In both species organophosphate poisoning causes cholinergic death only if AChE is highly inhibited. Further, in hens neuropathy is caused only if the maximum inhibition of NTE is greater than 70-80%. To evaluate the hen further as a model we need to know whether equally high inhibition of human NTE is a prerequisite of neuropathy in man. Autopsy material is required from cases of poisoning by compounds which are known to be able to cause neuropathy in sub-lethal doses. The degree of NTE inhibition in the lethal cases should give some guide to the threshold associated with neuropathy. Appeal was made for both NTE and AChE assays to be made whenever deaths occur from organophosphate poisoning; the causative agent should also be proven by tissue analysis and not presumed.

Lotti has carried out such an assay for a case of poisoning by the insecticide omethoate. Although AChE was highly inhibited there was no inhibition of NTE suggesting that neuropathic consequences are not to be expected from survivors of poisonings by omethoate in man. Lotti also showed that doses in excess of the LD₅₀ of omethoate did not inhibit brain NTE or cause neuropathy in hens either, again suggesting the hen may be a useful model.

Neural biopsies are obviously unsuit-

able for monitoring but R. J. Richardson (Ann Arbor) showed that the accessible blood lymphocytes contain an esterase identical to NTE in a variety of its biochemical properties. Provided that the natural fluctuation of lymphocyte levels can be allowed for, routine assay of lymphocyte NTE in potentially exposed persons such as spraymen may allow a neuropathy monitoring system analogous to the assay of blood AChE for acute poisoning.

At the LD₅₀ dose diethyl phosphates are always more likely to cause neuropathy than the analogous dimethyl esters. This can be rationalised by analysing effects on NTE independently of all other factors such as metabolic disposal. Study of structure/activity relationships for inhibition of NTE *in vitro* provides a basis for design of non-neuropathic anticholinesterase pesticides. For instance it has been found that esters with hydrophilic or heterocyclic leaving groups have comparatively little affinity for NTE in the test tube and low neuropathic potential *in vivo* and that insecticidal carbamates have no neuropathic potential.

In vivo phosphorylation of the neurotoxicity protein site by neuropathic esters occurs within a period ranging from a few minutes to two days after dosing depending on the route of administration and on whether they require metabolic activation. The secondary bond-cleavage is very rapid in most cases studied but there is then a silent period of about one week before axonal degeneration begins. A. Bischoff (University of Berne) reported that the earliest morphological changes are swellings of some rounded synaptic vesicles at excitatory nerve terminals. These changes were seen at a time when no such changes occurred in the flattened (inhibitory) vesicles although clinical signs of neuropathy were already apparent.

Toxicity testers are already reaping benefit from the understanding of the earliest events. As understanding is extended to the events in the silent period it is hoped that the processes by which axons are maintained may become clearer. Such understanding may be relevant to the modes of action of a wider range of neuropathic chemicals than only the organophosphorus esters. □

Laser polarization labelling

from Allister I. Ferguson

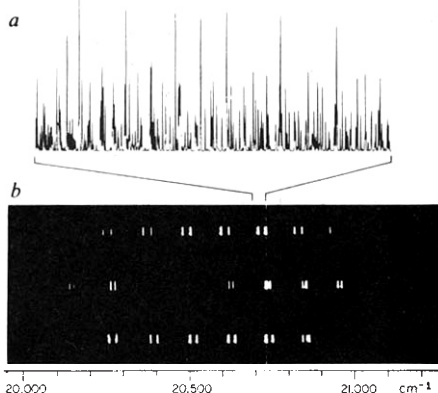
'A diatomic molecule is a molecule with one atom too many'. This definition, which was coined by Arthur L. Schawlow, reflects the kind of despair most spectroscopists encounter when attempting to unravel the complexities of molecular spectra. Even the simplest molecules have such a complicated structure that identifying and assigning quantum numbers to spectral lines is a major task. However, using a laser polarization labelling technique Schawlow and his colleagues at Stanford University have been able to simplify molecular spectra so that the spectra they obtain are more typical of atoms than of molecules. They have been able to identify new highly excited electronic states in a simple diatomic molecule and there are now good prospects for the application of the method to much more complicated molecules.

The factor which complicates molecular spectroscopy is that in addition to the electronic energy levels, which gives rise to atomic spectra, a molecule can vibrate and rotate. These vibrations and rotations add to the electronic energy levels changing a series of isolated levels into a complicated picture of overlapping bands. However, the Stanford group's technique enables a particular level within a band to be

'labelled' so that transitions from this 'labelled' level can be observed and a tremendous simplification in the spectrum be obtained.

The latest research has been reported by Nils Carlson, Toni Taylor and Arthur Schawlow (*Pys. Rev. Lett.* 37, 683; 1980). They studied the sodium dimer, Na₂, a widely studied diatomic molecule which, like the sodium atom in atomic physics, is much used for testing new techniques before they are applied to more complicated situations. Previously, only six electronic states of Na₂ were known but they have already reported the discovery of some fifteen new states and are confident of discovering even

Fig. 1a. A small portion of the absorption spectrum of Na₂. b. A larger portion of the same spectrum showing the simplification due to polarization labelling.



*A symposium on "Recent Developments in Mechanisms of Neurotoxicity" and a Round-Table discussion on "Delayed Neurotoxicity Testing of Organophosphates" were linked during the Second International Congress of Toxicology in Brussels, 6th - 11th July 1980.

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more in the near future. With a method powerful enough to find so many new states in such a well understood and highly studied molecule it is tantalising to speculate on the kind of discoveries which might be made on more exotic and less well understood molecules.

The technique that they use was first developed by Richard Teets, Richard Feinberg, Theodor Hänsch and Arthur Schawlow (*Phys. Rev. Lett.* 17, 683; 1976) and they called it laser polarization labelling. The method uses as a light source a narrowband tunable laser which can excite molecules from isolated vibrational-rotational levels of the ground electronic state to an electronically excited state. If the intensity of the laser is sufficiently large a significant proportion of the population of molecules in the lower level will be transferred to the upper level. By choosing the polarization of the laser appropriately it is possible to orient the levels of the transition in such a way as to create an optical anisotropy in the medium. These levels are said to be 'labelled'. This anisotropy will be experienced by light which is resonant with one of the 'labelled' levels and some other level. Now if broadband light is passed through the medium most of the light will be transmitted without change in polarization. However those light frequencies which do resonate with one of the 'labelled' levels and another level will suffer a depolarization. This depolarization can easily be detected by passing the light through a crossed polarizer. Only those frequencies which interacted with the 'labelled' levels will be transmitted and the spectrum of the this light can be recorded with a spectrograph.

The number of levels that can be reached from a particular 'labelled' level is quite limited due to the quantum mechanical selection rules. In fact, for each 'labelled' level there are at most three rotational levels which can be accessed for each vibrational state. Further selection rules often reduces this number to two.

A typical polarization labelled spectrum is shown in Fig. 1. This shows a small part of the spectrum of Na_2 . The pairs of lines in Fig. 1 b correspond to the two allowed transitions from each 'labelled' level for each vibrational state. These are the P and R branch transition in the terminology of molecular spectroscopy. Each pair of lines correspond to transitions to different vibrational levels of the excited electronic state. By scanning a wide enough spectral region it is possible to identify the lowest vibrational level and the vibrational and rotational quantum numbers can be found. Given these the molecular constants for the electronic state can then be easily calculated. The classification of the electronic state is determined by selection rules. Also shown in figure 1 a for comparison is a traditional absorption spectrum of a much smaller portion of the Na_2 spectrum. Clearly identification of particular electronic states and vibrational

and rotational quantum numbers from such a spectrum is extraordinarily difficult.

Carlson *et al.* have scanned wide regions of the Na_2 spectrum and have managed to identify no fewer than fifteen new electronic states. Ten of these states are characterised as being $^1\Delta_g$ and five as $^1\pi_g$. The energies of these new states have been found to follow the well known Rydberg formula, with small corrections due to molecular symmetry. The states are found to correspond to principal quantum numbers of $n=6-15$ for the $^1\Delta_g$ states and $n=4-8$ for the $^1\pi_g$ states. It should be possible to also find new Σ states and the Stanford group have begun the search for

these.

The study of highly excited molecules (Rydberg molecules) has been severely hampered by the very complex structure of the closely spaced Rydberg molecular levels. At the same time studies of Rydberg atoms have been very fruitful (Metcalf *Nature* 284, 127; 1980) — so much so that Rydberg spectroscopy is now a sub-branch of atomic physics. The work of Carlson *et al.* is a breakthrough in unravelling and interpreting the Rydberg molecule and is the first step to a new and fuller understanding of highly excited molecules which should open the way to Rydberg molecular spectroscopy. □

Combination chemoprevention of cancer

from Michael B. Sporn

THE current use of cytotoxic drugs for treatment of invasive cancer in man depends largely on combination chemotherapy — the combined use of several drugs which exert synergistic effects to achieve the goal of total tumour cell kill. Several papers presented at a recent meeting* suggest that for similar reasons 'combination chemoprevention' might be of importance in the future development of non-cytotoxic drugs for prevention of cancer, that is, for the arrest or reversal of the development of neoplasia in its pre-malignant stages. Recent advances in the understanding and control of tumour progression (promotion) have been particularly striking. Using the classical two stage model of mouse skin carcinogenesis (the hydrocarbon, DMBA, as initiator, and the phorbol ester, TPA, as promoter), A.K. Verma and R.K. Boutwell (University of Wisconsin) reported that the combinations of either retinoic acid plus the steroid, dexamethasone, or retinoic acid plus the protease inhibitor, TLCK, exert marked synergistic preventive effects. Their results suggest that retinoic acid, dexamethasone, and TLCK inhibit skin tumour promotion by independent mechanisms. Similar conclusions were reached recently by T.J. Slaga *et al.* (Oak Ridge National Laboratory; *Proc. natn. Acad. Sci. U.S.A.* 77, 2251; 1980) who have shown that the promotion stage in this system can be further divided into two stages, with the first stage caused by limited application of TPA, and the second caused by the related weak diterpene promoter, mezerein. Slaga *et al.* suggest that low doses of TPA induce formation of primitive skin stem cells, while mezerein

induces formation of polyamines and resultant cell proliferation. The TPA effect can be blocked by the protease inhibitor, TPCK, while the mezerein effect is blocked by retinoic acid; glucocorticoids apparently inhibit both stages of promotion. In other studies, Verma, Ashendel, and Boutwell (*Cancer Res.* 40, 308; 1980) have shown that retinoic acid and indomethacin, an inhibitor of prostaglandin synthesis, exert a synergistic effect to block the induction of ornithine decarboxylase (ODC) caused by TPA; ODC activity has been previously implicated as a key step in formation of polyamines. Whether retinoids and inhibitors of prostaglandin synthesis are synergistic for prevention of skin tumours in the mouse is currently being studied.

Another important area for studies of initiation and promotion is mammary carcinogenesis, in which the internal hormonal milieu of the organism is believed to provide the required promotional stimulus for development of cancer. In the female rat, one or two doses of the alkylating carcinogen, nitrosomethylurea, suffice to initiate carcinogenesis. In new studies, C.W. Welsh (Michigan State University) and R.C. Moon (IIT Research Institute) reported that the combined use of a suppressor of prolactin secretion (2-bromo- α -ergocryptine) together with the retinoid, retinyl acetate, was distinctly superior to either treatment alone in preventing mammary cancer after rats were treated with the carcinogen. Prolactin has long been suspected as a promoting agent in human mammary carcinogenesis, and suppression of its secretion has an obvious rationale. The molecular mechanism of action of the

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*Annual Meeting of the American Association for Cancer Research, San Diego, California, May 28-31, 1980.

retinoids in this system is not known, although it has been shown (Moon *et al.* *Cancer Res.* **39**, 1339; 1979) that they have an anti-proliferative effect on mammary epithelium.

Combination chemoprevention also is applicable to inhibition of the initiation of cancer, as was reported by P.W. Dion, W.R. Bruce *et al.* (Ontario Cancer Institute). It had previously been shown by Bruce's group that mutagenic substances (assayed by the Ames test) are found in human faecal material, and they have been suggested as possible endogenous causative agents for colon cancer. Since it is suspected that these mutagenic substances may be N-nitroso derivatives of intestinal lipids, Bruce *et al.* have fed ascorbic acid and α -tocopherol (known inhibitors of nitrosamine formation) to human volunteers. They reported that the combination of ascorbic acid and tocopherol reduced the median level of faecal mutagen to 25% of control values; the rationale for the combination is to achieve an effective level of anti-oxidant in both the aqueous and hydrophobic phases of the intestinal contents. Another important observation relating to ascorbic acid and cancer prevention was reported by W.F. Benedict and P.A. Jones (University of Southern California), who have shown that levels of ascorbic acid as low as 1 μ g per ml can

inhibit malignant transformation of mouse fibroblasts caused by methylcholanthrene in cell culture. They found that ascorbic acid was effective in blocking transformation when given as late as 3 weeks after exposure of cells to the carcinogen. Previously, ascorbic acid had been considered to be of chemopreventive use only as an anti-initiating agent. Benedict and Jones' new studies (see also *Cancer Res.* **40**, 2796; 1980) suggest that ascorbic acid may also function as an anti-promoting agent, and will undoubtedly add further interest to the already controversial area of the use of this compound in treatment and prevention of malignancy.

Whatever may be the outcome of these studies, the field of combination chemoprevention appears to be developing rapidly. In other areas of pharmacology the use of multiple drugs to achieve synergistic action and to diminish toxicity is well established. At present, it cannot be predicted how successful such methods might be for applied chemoprevention in man. However, as interest in pharmacological prevention of cancer increases (and as concern about the possible toxic effects of some of the preventive agents, particularly the retinoids, also increases), combination chemoprevention will undoubtedly attract further attention. $\square$

changing T to a G in the TATAA box lying within the promoter region also reduces the rate of initiation substantially.

Mathews (Cold Spring Harbor) concluded from the hybridization experiments that the non-coding Ad-2 VA RNAs may play a role in virus messenger RNA splicing. Flint (Princeton University) using antibodies from patients with systemic lupus erythematosus which immunoprecipitate different classes of human ribonucleoproteins reported that some of these antisera inhibit the splicing of Ad-2 early RNA in nuclei isolated from cells infected for 8 hours in the presence of cytosine arabinoside.

Another *in vitro* system that continues to yield intriguing information is that for DNA synthesis. Kelly (Johns Hopkins University), Stillman (Cold Spring Harbor) and Winnacker (Munich University) showed that nascent DNA chains initiated *in vitro* have terminal protein covalently attached to the 5' end. Kelly further showed that the polypeptide attached *in vitro* has a molecular weight of 80K in contrast to the 55K product seen on mature virion DNA. However, pulse chase experiments *in vivo* suggest that the 80K form may be a precursor of the 55K protein. Furthermore, they give the same patterns of partial proteolysis with chymotrypsin and are attached to the DNA by the same linkage; a phosphodiester bond between the β -OH group of serine and the 5'-OH of the terminal deoxycytidine in the DNA.

By stringently inhibiting host cell protein synthesis with anisomycin Lewis (Cold Spring Harbor) reported that his group have identified a new class of 'immediate early' Ad-2 proteins. One of these proteins, which is encoded between 17.0 and 21.5 map units, was clearly shown to be synthesised independent of 'early' region 1A.

Evidence was presented by a number of groups that the Ad-2 major late promoter (co-ordinate 16.4) functions 'early' after infection in permissive cells. Ziff (Rockefeller University) demonstrated by RNase T-1 finger printing that the capped undecanucleotide and promoter proximal spots 1-4 could be clearly identified in cycloheximide treated cells 2-4 hours post infection. Lewis and Akusjarvi (Uppsala University) both identified *in vitro* synthesised proteins (52K and 55K) using Ad-2 m-RNA transcribed from the major late promoter in drug blocked infected cells. Akusjarvi also reported that both of these proteins are synthesised *in vivo* in infected cells blocked for DNA synthesis using cytosine arabinoside.

This meeting produced no definitive data to clarify which adenovirus gene(s) or products are responsible for transformation or malignancy. Past failure to isolate adenovirus conditional lethal mutants defective for transformation has encouraged several groups (Williams, University of Pittsburgh; Ginsberg,

Adenovirus complexities

from correspondents

Two years ago splicing was the hottest subject of discussion at the EMBO workshop on the molecular biology of adenoviruses but the latest meeting* showed that many of the details of complex splicing patterns have already been untangled. Now, the control of virus transcription and DNA replication remain to be elucidated and there has been a clear trend to the development of *in vitro* systems to dissect further the intricacies of eukaryotic cellular control mechanisms.

In vitro transcription systems derived from uninfected KB or HeLa cells with purified RNA polymerase have been used to define the requirements for specific *in vitro* initiations of viral transcription (Lee, University of St. Louis and Corden, University of Strasbourg). Lee used cloned viral DNA fragments as templates to show that the major late promoter is used to initiate RNA products in the correct direction and of the size predicted from the size of the DNA fragment used. All other *in vivo* promoters are also active *in vitro* with the notable exception of the E2 promoter at 75 map units; it is known from sequence analysis that this site lacks a recognisable

'TATAA' box. The promoters vary in strength in the *in vitro* system over a tenfold range, with the major late promoter being most active and early E1A the least active. By using as template a DNA fragment that included the whole of the polypeptide IX transcription unit (the sequence of which is known and which is not spliced *in vivo*), Lee also showed that in his system at least, termination does not occur at a specific site. Transcription continues through the known poly A addition site and very large RNA products are obtained.

Corden, using a similar *in vitro* system, has introduced specific deletions into the DNA template in the region of the major late promoter, to define the sequence requirements for specific initiation of transcription. Removal of bases more than 31 positions upstream from the cap site, or after the site of initiation, did not inhibit *in vitro* transcription, but removal of bases at -31 or -30 resulted in a five-hundred fold reduction in initiation. Similar experiments defined the 5' promoter boundary at -12 relative to the cap site. In analogous experiments using cloned conalbumin promoter DNA, Corden showed that

*The meeting was held at Peebles, Scotland, June 14-18, 1980.

Columbia University and Jones, Purdue University) to use molecular biology techniques and/or *in vitro* mutagenesis to create new mutants. Williams described a 'cold sensitive' mutant which apparently lacks transforming ability. He also reported that the ability of a virus to stimulate host cell DNA synthesis was not alone sufficient to induce transformation. Jones reported studies on an Ad-5 mutant that lacked a single transcript from early 1A which they had generated by inserting an octanucleotide (GGAATTCC) into the Sma I site at 2.85 map units. Preliminary data suggest that this insertion affects transformation.

A number of papers were presented which clearly show that adenovirus DNA is integrated into rodent DNA by illegitimate recombination events. Sambrook (Cold Spring Harbor) Ad-2, Visser (Utrecht University)

Ad-5 and Stabel (Cologne University) Ad-12, provided data that for each transformed cell line studied viral integration had occurred at different sites in the host genome. Van den Elsen (University of Leiden) reported that the oncogenicity of Ad-5 and Ad-12 transformed baby rat kidney cell lines in nude mice required the expression of the entire 'early' regions 1A and 1B. Cell lines not expressing 1B 65K protein (Ad-5) or 1B 60K protein (Ad-12) were found to be non-tumorigenic. Graham (McMaster University), reporting on similar experiments using baby hamster kidney cells, concluded that in this system tumorigenic cell lines could be obtained by transfection of Ad-5 DNA which represented early region 1A, but only part of early region 1B (HIND III G fragment).

Raska (CMDNJ — Rutgers) using both serum and cell mediated cytotoxic tech-

niques *in vitro* demonstrated that Ad-12 'early' region 1 codes for a membrane associated antigen. This may be the bonafide virus specific transplantation antigen. However, it is also known that some transformed rat cell lines have a membrane glycoprotein bound to the major histo-compatibility antigens. The precursor virus protein of the membrane bound glycoprotein is 16K and is encoded in early region 3.

In conclusion, it seems likely that the next two years of adenovirus research will be dominated by molecular engineering and *in vitro* systems with *in vitro* mutagenesis providing the geneticists with a powerful tool to define more clearly which gene(s) is responsible for malignant transformation and which proteins are involved in transcriptional control of this complicated unit of genes. □

Polymerase III control region defined

from Peter J. Ford

RECENT experiments show that in at least one class of eukaryote genes — 5S ribosomal RNA genes transcribed by RNA polymerase III — control of RNA synthesis is quite different from that found in prokaryotes. In a typical prokaryote gene a promoter site, which binds RNA polymerase, is situated about 10 base pairs upstream (to the 5' side) from the site at which transcription begins. Mutations in the promoter site alter the level of gene expression but do not affect the actual control of expression. A second site, the operator, overlapping the polymerase binding site is found about 20 base pairs downstream (to the 3' side) of the initiation site. The operator binds a repressor molecule which prevents polymerase from binding to promoter. Control is exercised by the interactions of small molecules which facilitate either the removal or the binding of the repressor to the operator site. Thus the prokaryote gene consists of a regulatory site overlapping a polymerase binding site located at or just upstream from the point at which transcription begins.

In prokaryotes all genes are transcribed by a single RNA polymerase. In eukaryotes, however, there are at least three structurally and functionally distinct RNA polymerases. Polymerase I transcribes only ribosomal RNA, polymerase II transcribes heterogeneous nuclear RNA (mRNA precursors) and polymerase III transcribes a variety of low molecular weight RNAs including transfer RNAs, 5S ribosomal RNA, low molecular weight RNAs and viral-associated low molecular weight RNAs. The small size of the Polymerase III

primary transcripts makes their identification easy and this coupled with their lack of post-transcriptional processing has made genes transcribed by Polymerase III the first choice for studies of regulation.

The somatic 5SRNA gene of *Xenopus borealis* was chosen by Sakonju, Bogenhagen and Brown (*Cell* 19, 13; 1980) in their search for the eukaryote promoter site. A single 5S gene copy cloned in pBR322 can promote faithful transcription (correct initiation and termination) of 5S RNA in an *in vitro* and an *in vivo* (oocyte injection) assay. By an *in vitro* genetic engineering procedure regions flanking both 5' and 3' ends of the gene were deleted and the deleted genes recovered after reinsertion into pBR322. Deletion end points were determined by sequencing the DNA and were numbered from the transcription initiation site (position +1), base pairs downstream (within the gene) being numbered +2, +3, ... and base pairs upstream being numbered -1, -2 -3 and so on.

Genes with deletions extending into them from the 5' end were tested for transcription activity in an *in vitro* transcription assay (Birkenmeier, Brown & Jordan *Cell* 15, 1977; 1978). It was shown that genes with all 5' flanking sequences deleted up to the initiation site are actively transcribed but begin at an incorrect site a few nucleotides into the gene, suggesting that the 5' flanking sequence is required for correct initiation but does not control transcription. Deletions were made extending further into the gene but not until 50 base pairs had been removed was there a drop in transcription efficiency. Remarkably, the size of the transcripts remained about 120 nucleotides, the size of normal 5SRNA. Thus polymerase III is recognizing a sequence starting at least 50 base pairs into

the gene from the normal initiation site and is beginning transcription on any appropriate base pair in the region where the normal initiation site should have been put even though this is now replaced by vector pBR322 DNA.

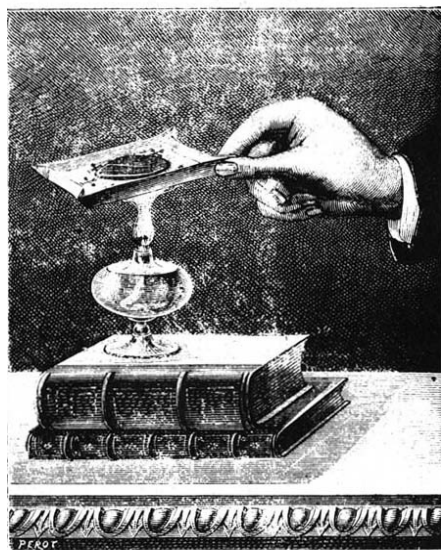
Two points are evident — polymerase binding and transcription depend on a site within the gene itself while the exact point of initiation is influenced by the sequence surrounding the initiation site. To test the relative importance of these two effects Sakonju *et al.*, constructed two 'maxigenes' which had an extra 6 or 20 base pairs inserted between positions +40 and +41 of the gene sequence. Maxigenes are transcribed to give 5S sized transcripts which have begun transcription at sites within the gene. For the +6 maxigene the initiation point is at +10 or +11 base pairs and for the +20 maxigene the initiation point is at either position +21, +22, +23 or +24 within the normal gene sequence. Thus the site within the gene is dominant over the sequence at the normal initiation site, and acts to promote initiation at any convenient site at a loosely fixed distance (about 50 base pairs) upstream from that site, the precise initiation point being determined by the local sequence(s) surrounding potential initiation sites.

Transcription experiments were also carried out with genes deleted progressively from the 3' end (Bogenhagen, Sakonju & Brown *Cell* 19, 27; 1980). Since deletions of the 3' end lack a correct termination site a technique for identifying correct initiation in the absence of correct termination had to be used. Cordycepin triphosphate (3' dATP) was included in the *in vitro* assay mix serving to terminate a proportion of transcripts at each A residue in the gene sequence. Correct initiation generates a defined set of molecules (analogous to a sequencing ladder) which can be



100 years ago

PHYSICS WITHOUT APPARATUS



Melting lead on a playing card

A pretty experiment which is easily performed is that of boiling water in a sheet of paper. Take a piece of paper and fold it up, as schoolboys do, into a square box without a lid. Hang this up to a walking-

stick by four threads, and support the stick upon books or other convenient props. Then a lamp or taper must be placed under this dainty cauldron. In a few minutes the water will boil. The only fear is lest the threads should catch fire and let the water spill into the lamp and over the table. The flame must therefore not be too large. A small taper will give a flame quite large enough. The paper does not burn, because it is wet; and even if it resisted the wet it still would not burn through, because the heat imparted to it on one side by the flame would be very rapidly conducted away by the water on the other. Another experiment of a similar nature, but perhaps even more striking, is as follows: — Twist up the edges of a common playing-card or other bit of eardboard, so as to fashion it into a light tray. On this tray place a layer of small shot or bits of lead, and heat it over the flame of a lamp. The lead will melt, but the card will not burn. It may be charred a little round the edges, but immediately below the lead it will not be burned, for here again the lead conducts off the heat on one side as fast as it is supplied on the other. Lastly, we give an experiment which, like the two preceding, proves that a good conducting substance may protect a delicate fabric from burning by conducting away the heat rapidly from it. Lay a piece of muslin quite flat upon a piece of metal. A live coal placed on the muslin will not burn it, for the metal takes away the heat too fast. If the muslin is however laid on a bad conductor, such as a piece of wood, it will not be protected, and the live coal will kindle the muslin.

A certain way to obtain an electric spark we have found with what we may call a *Tea-tray Electrophorus*. A common tea-tray of metal is supported on two dry glass tumblers. A piece of common brown paper cut so as to be a little smaller than the tray, and with rounded corners, is warmed, laid on the table and rubbed briskly with a piece of indiarubber, or with a clothes-brush. It is then laid

down for an instant on the tray and the tray is touched with the hand. The brown paper is then lifted a few inches above the tray. If at this juncture some person presents his knuckle to the tray he will receive a bright spark, which under favourable circumstances may be a couple of inches long. By simply putting the paper down, touching the tray,



The Tea-tray Electrophorus.

and again lifting up the paper the tray is again charged: and a large number of sparks may be thus drawn one after the other in rapid succession. The paper may be lifted by the hands, but it will be found better if a couple of ribbons or strips of paper be fixed on with wax to serve as handles, as shown in our figure.

From *Nature* 22, 9 & 16 September, 440 & 464, 1880.

recognized by their electrophoretic mobility in acrylamide gels. By such means the 3' end of the gene could be deleted to between +80 and +83 of the gene sequence before correct initiation failed. Together with the previous result this defines the region in *X. borealis* somatic 5S DNA which is required for transcription as lying between positions +50 and +83 of the gene sequence.

An internal promoter is certainly unexpected and different in location from the 5' flanking promoters of bacterial genes, but is it a general rule for all polymerase genes? The answer is probably yes since there is significant homology of a sequence AGCAGGGT found between base pairs +55 and +62 of the 5S gene and a similarly (± 2 base pairs) located sequence in other polymerase III genes — Adenovirus VA I gene and *Bombyx mori* tRNA^{met}.

Moreover, similar deletion and transcription studies on tRNA genes (Telford *et al. Proc. natn. Acad. Sci. U.S.A.* 76, 2590; 1979) show that deletion of 5' flanking sequences to within -20 base pairs of the transcription initiation site does not impair transcription of the *Xenopus laevis* tRNA^{met} gene. However, a mutation (Ad5d1309, a dimer deletion) occurring outside the gene between pairs -21 and -24

of the 5' flanking sequence of adenovirus 5 VA I gene does prevent synthesis of one of the two alternate VA I RNAs (Thimmappaya, Jones & Shenk, *Cell* 18, 947; 1979). The two VA I RNAs are both transcribed from the same gene but differ by three nucleotides at the 5' end, VA IA begins with pAGCG . . . while VA IG begins at the second G. The Ad5d1309 mutant produces only the VA IG RNA. The two base pair deletion thus alters the 5' flanking sequence sufficiently to suppress initiation at A but not at G, demonstrating, in an *in vivo* situation, that local sequences influence the exact site at which transcription begins.

Clearly if the same polymerase III enzyme transcribes a wide variety of genes some of which are independently regulated there must be other factors interacting with the gene-polymerase complex to regulate transcription. One such factor has recently been purified from soluble extracts of *Xenopus* ovaries (Engelke, Ng, Shastry, & Roeder *Cell* 19, 717; 1980). It had been noticed that 5S genes in chromatin from oocytes were transcribed by a purified polymerase III preparation but a cloned 5S gene was not (Ng, Parker & Roeder *Proc. natn. Acad. Sci. U.S.A.* 76, 13; 1979). Activity could be restored to the purified polymerase III/cloned 5S gene system by

addition of an ammonium sulphate fraction of soluble ovary proteins. Such restoration assays made it possible to purify a 37,000 molecular weight protein which allows faithful transcription of both *Xenopus* somatic and oocyte cloned 5S genes in a defined reconstituted transcription system using purified polymerase III. The factor, which appears specific for 5S genes, has no activity on a cloned *Xenopus* tRNA^{met} gene. The factor binds independently of polymerase III to a region of the 5S gene between +45 and +96 base pairs completely overlapping the region identified by deletion mapping as essential for transcription in 5S DNA. Thus there is a possibility that this factor acts in a positive way by directing polymerase III to the 5S gene. It seems certain that this is the first of many factors which will be found to specifically modify the interaction between polymerase III and its genes.

Thus the dogma generated by studies of gene regulation in prokaryotes is breaking down with the demonstration of an internal promoter and regulatory sites for 5S RNA transcription in *Xenopus*. These studies have not yet defined the nature of developmental regulation of these genes, but they have gone a long way towards that goal. □

ARTICLES

Message sequences and short repetitive sequences are interspersed in sea urchin egg poly(A)⁺ RNAs

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At least half of the mass and most of the different single-copy maternal mRNA sequences in the egg are covalently associated with transcripts of short repetitive sequences. Only a restricted group of the diverse genomic repeat families are significantly represented. The messages fall into several hundred sets, each containing transcripts from a different repeat family.

THE sea urchin genome includes over 10^5 short repetitive sequence elements interspersed among the single-copy DNA regions¹. Renaturation kinetics^{1,2}, as well as primary sequence data³, indicate that the genomic repeats belong to several thousand distinct sequence families, which in general share little or no homology. Although the biological role of these sequences is unknown, recent studies have shown that almost all of the diverse repeat families are represented in a tissue-specific manner in sea urchin nuclear RNAs⁴ and in unfertilized egg RNA⁵. The present work was undertaken to determine the nature of those repeat transcripts stored in the egg. We found that many of these transcripts are covalently associated with RNAs which have the characteristics of egg maternal messengers. Thus, repetitive sequences are interspersed in egg poly(A)⁺ RNA [p(A)⁺ RNA] and are included in a large majority of the diverse species of egg message.

Repeated sequence transcripts are included on many egg poly(A) mRNA molecules

According to earlier studies⁵ most of the transcripts of short interspersed repetitive DNA sequences in the sea urchin egg are present on RNA molecules several thousand nucleotides long. The possibility thus arose that the repeat sequence transcripts are located on maternal message molecules. The egg of *Strongylocentrotus purpuratus* contains about 50 pg of maternal mRNA (about 1.5% of the total egg RNA) bearing poly(A) tails sufficiently long to promote binding to oligo(dT)-cellulose columns⁶⁻⁸. We refer to the egg RNA collected on such columns as the 'p(A)⁺ RNA', although the flowthrough or 'p(A)⁻ RNA' fraction apparently includes an approximately equal mass of maternal message, much of which is terminated by short 3' oligo(A) tails which bind to poly(U)-Sepharose and not to oligo(dT)-cellulose⁶⁻⁹. Isolation of the p(A)⁺ fraction of the egg RNA provides a convenient experimental enrichment for at least a large subclass of the maternal message. In addition, use of this fraction minimizes the contribution of known repetitive gene transcripts such as the histone mRNAs, which, in the sea urchin, do not co-purify with p(A)⁺ RNA¹⁰.

The p(A)⁺ RNA of the egg was isolated and purified by successive rounds of oligo(dT)-cellulose chromatography. The denatured weight-average length of the p(A)⁺ RNA was about 5,000 nucleotides, in general agreement with previous

measurements^{7,11}. This was determined by electron microscopy and gel electrophoresis as described below. The concentration of repetitive sequence transcripts was examined in the p(A)⁺ RNA and the p(A)⁻ RNA fractions. Figure 1a and b displays titration reactions with two cloned repetitive sequences, CS2109B and CS2108, shown earlier to be represented by prevalent egg transcripts⁵. The cloned probes were labelled and strand-separated, and one strand of each was reacted with increasing amounts of egg p(A)⁺ RNA or p(A)⁻ RNA⁴. Transcripts complementary to the CS2109B sequence (Fig. 1a) are 32-fold more concentrated in p(A)⁺ RNA than in p(A)⁻ RNA, and a similar degree of concentration, 38-fold, is observed for the CS2108 sequence (Fig. 1b). Given that 1.5% of egg RNA is included in the p(A)⁺ RNA fraction, these results require that at least a third of the RNA molecules reacting with the two cloned repeats contain long poly(A) tracts.

To determine whether these two cloned repeats are representative, we measured the kinetics of the reaction of a ³H-DNA genomic repetitive sequence tracer with excess egg p(A)⁺ RNA, p(A)⁻ RNA and total egg RNA. As shown in Fig. 1c, all three reactions were heterogeneous in rate, due to variation in the prevalence of different repeat transcripts⁵. However, it is clear that transcripts of most repeat families detectable in egg RNA are concentrated in the p(A)⁺ RNA fraction. Considering the initial 30% of each reaction, which represents the more prevalent repeat transcripts, the reaction with p(A)⁺ RNA occurs about 33 times more rapidly than with unfractionated egg RNA. Hybridization with p(A)⁻ RNA is not detectably slower than with total egg RNA, so not all repeat-containing molecules are included in the p(A)⁺ RNA fraction. It can be calculated from these data that about 50% of the transcripts of each average prevalent repeat species co-purifies with the p(A)⁺ RNA fraction. For comparison, the distribution of single-copy sequence transcripts in the p(A)⁺ RNA and p(A)⁻ RNA of the egg is shown in Fig. 1d. The tracer in this experiment is a fraction of single copy ³H-DNA selected by prior reaction with egg RNA ('egg DNA', or 'eDNA')¹². Analysis of the kinetics shows that roughly half of the diverse species of single-copy sequence transcript present in the egg are heavily represented in the p(A)⁺ RNA fraction. That is, an average of about 75% of the molecules of each RNA species in this class is recovered as p(A)⁺ RNA. The other half of the mRNA species is mainly found in the p(A)⁻ RNA fraction, though a small percentage of these molecules is included in the p(A)⁺ RNA fraction, so that the two reactions ultimately terminate together. Of course, many RNA species may have

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intermediate degrees of polyadenylation. Additional evidence has been obtained with cloned single-copy DNA probes. As reported elsewhere¹³, about 50% of the prevalent transcripts of cDNA clone SpG30 are found in the egg p(A)⁺ RNA fraction. Similarly, about 35% of the rare transcripts of the cloned Sp88 single copy sequence are recovered as p(A)⁺ egg RNAs¹⁴.

The distribution of repeat-sequence transcripts in p(A)⁺ and p(A)⁻ RNA fractions is consistent with the view that the repeats are found on p(A)⁺ mRNA molecules. Thus, at least for those repeat families which are represented by prevalent egg RNA transcripts, about half of the molecules bearing each repeat sequence are recovered in the p(A)⁺ RNA fraction. Furthermore, note that all the diverse repeat transcripts appear to be relatively concentrated in the p(A)⁺ RNA fraction, while only about half of the different species of single-copy sequence transcript are recovered to a large extent as p(A)⁺ RNAs.

Electron microscopy of renatured egg p(A)⁺ RNA

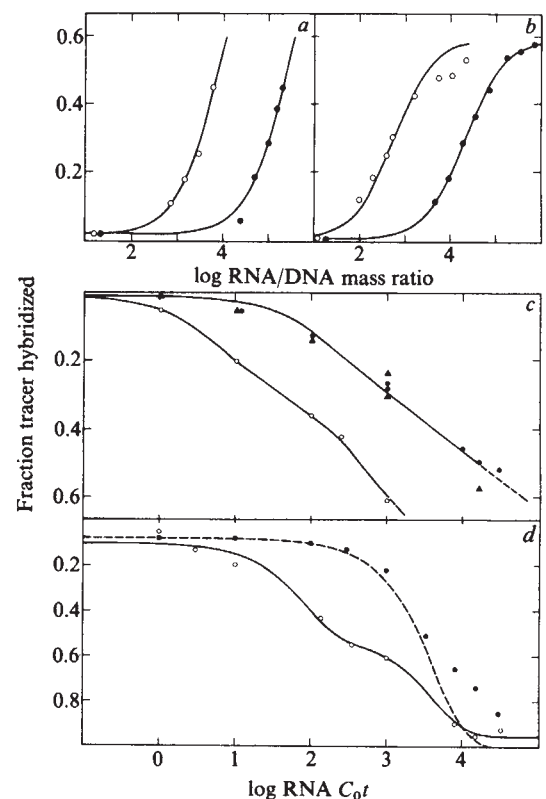
A dramatic visualization of the interspersed sequence organization of egg p(A)⁺ RNA is presented in Fig. 2. These electron micrographs illustrate the multi-stranded branched structures formed when p(A)⁺ RNA molecules are renatured. The RNA was first fully denatured, then incubated to an equivalent C_0t of 600 Ms in a buffered formamide medium and spread for electron microscopy, as described in the legend to Fig. 2. Almost no complicated structures, such as those shown, were observed in control samples spread immediately after denaturation ($C_0t < 2 \times 10^{-3}$ Ms).

Interpretation of the structures shown in Fig. 2 is greatly assisted by the previous observation⁵ that both complements of

each repeat sequence are represented in sea urchin egg RNA, as in nuclear RNAs⁴. This is almost certainly not due to symmetrical transcription but to the presence of RNA molecules deriving from different transcription units orientated oppositely with respect to the repeat sequence. In contrast, all single-copy sequences so far analysed in sea urchin egg or nuclear RNAs are asymmetrically represented. This includes a number of specific sequences complementary to cloned probes (ref. 15 and unpublished data), as well as the total single-copy sequence sets of nuclear RNA¹⁶. With this background, it is clear that the multi-stranded structures shown in Fig. 2 are networks consisting of several individual long p(A)⁺ RNA molecules held together by renatured complementary short repeat duplexes. The structures shown bear a strong resemblance to the structures formed when DNA from an animal displaying short-period repetitive sequence interspersion is renatured at low C_0t (ref. 17). RNA networks held together by renatured interspersed repeats were described previously by Federoff *et al.*¹⁸, who observed them in HeLa cell nuclear RNA.

About 65% of the total contour length of the p(A)⁺ RNA in a random sample studied (1.2×10^6 nucleotides) is included in branched structures displaying four or more ends, and apparently composed of two or more molecules. This should approximately estimate the mass fraction of p(A)⁺ RNA-bearing repeats, since most of the RNA contour length appears to consist of single-stranded regions. Although it is impossible to interpret most of the RNA complexes unambiguously, some qualitative implications follow from their general structure. The presence of complementary repeat sequence transcripts previously observed in total egg RNA is seen to be a property of the p(A)⁺ RNA. Over two-thirds of the molecules participating in intermolecular duplexes were present in structures displaying six or more ends, and therefore consisting of at least three

Fig. 1 Distribution of repetitive and single-copy sequences in egg p(A)⁺ RNA and p(A)⁻ RNA fractions. Total egg RNA was isolated³⁵ and fractionated on oligo(dT)-cellulose⁴⁰. The RNA which bound consecutively on three passes was the p(A)⁺ RNA fraction (1.5% of total egg RNA). RNA which repeatedly failed to bind was the p(A)⁻ RNA fraction (98%). All RNAs were partially hydrolysed with alkali to about 1,000 nucleotides before hybridization. *a*, Titration of p(A)⁺ and p(A)⁻ RNA with the cloned repetitive-sequence, CS2109B²⁴. The repetitive sequence cloned in plasmid CS2109B was excised with *Eco*RI, 5' end-labelled with ³²P and strand separated⁴. Excess lower strand tracer was hybridized to termination with increasing amounts of p(A)⁺ (○) or p(A)⁻ (●) RNA. All hybridization reactions described here were assayed by hydroxyapatite binding, unless otherwise noted. The curves represent least-squares solutions to the form for the titration reactions given in equation (2) of Scheller *et al.*⁴. The specific fraction of p(A)⁺ RNA consisting of CS2109B transcripts was 1.3×10^{-4} , and for p(A)⁻ RNA the fraction was 4.1×10^{-6} . *b*, Titration of p(A)⁺ RNA and p(A)⁻ RNA with the upper strand of the cloned repetitive sequence CS2108²³. The specific RNA fractions measured are 1.8×10^{-3} for p(A)⁺ RNA and 4.7×10^{-5} for p(A)⁻ RNA. *c*, Hybridization of excess p(A)⁺ RNA (○), p(A)⁻ RNA (●) or total egg RNA (▲) with repetitive ³H-DNA tracer representing most of the short repeats in the genome. This tracer was prepared, as previously described⁵, by reassociating sea urchin ³H-DNA fragments of average length 3,300 nucleotides to C_0t 40 Ms, digesting with *S*₁ nuclease, binding to hydroxyapatite, and fractionating according to molecular size on a Sepharose CL-2B column. The resulting short-repeat tracer displayed a weight-average length of 300 nucleotides. Hybridization was at 50 °C in 0.12 M phosphate buffer, pH 7.0. The data were fitted assuming second-order kinetics^{4,5} with two kinetic components, but since the reactions are not terminated, only the faster component of each reaction, representing the more prevalent repeat transcripts, is meaningful. A fast component terminating at 30% hybridization was arbitrarily selected for analysis. The observed rate constants for this component are: p(A)⁺ RNA, $k = 1.54 \times 10^{-1} \text{ M}^{-1} \text{ s}^{-1}$; total egg RNA or p(A)⁻ RNA, $k = 4.6 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$. *d*, Hybridization of excess p(A)⁺ RNA (○) or p(A)⁻ RNA (●) with ³H-labelled eDNA tracer. The eDNA tracer was prepared by enriching for the fraction of total single-copy ³H-DNA that hybridizes with total egg RNA (about 3%). This particular eDNA tracer hybridized to an extent of 45% with total egg RNA and was thus about 15-fold purified relative to the starting single-copy DNA. Reactions with p(A)⁺ and p(A)⁻ egg RNAs have been normalized to the 45% terminal value. The dashed line indicates the kinetics of the reaction of eDNA with total egg RNA, that is, a single pseudo-first-order kinetic component with a rate constant of $2.3 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$. This rate was previously determined with several eDNA preparations¹² (see also Fig. 6a). The reaction with p(A)⁺ RNA is heterogeneous and is fitted by two components (solid line), a fast component representing 41% of the tracer, with a rate constant of $1.2 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$, and a slow component representing 43% of the tracer, with a rate constant fixed at $2.3 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$. Comparing these rate constants to the rate constant for total egg RNA, we calculate that the fast component represents sequences 75% of whose transcripts are recovered in the p(A)⁺ RNA fraction, while the slow component represents sequences that are mainly (>95%), though not exclusively, confined to the p(A)⁻ RNA fraction. Note that the sequence concentration of almost half of the single-copy species is lower in the p(A)⁻ RNA than in total egg RNA. These are presumably the species predominantly found in the p(A)⁺ RNA fraction.



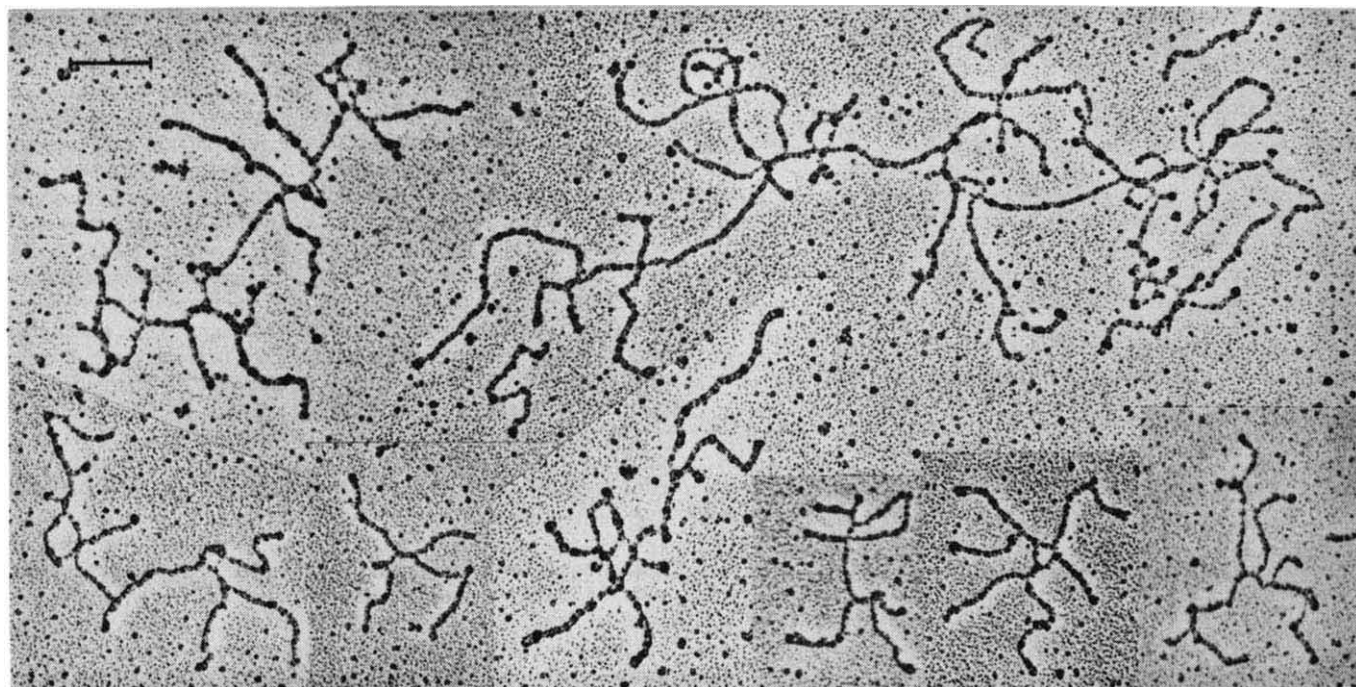


Fig. 2 Electron microscopy of multimolecular structures formed by renaturation of egg p(A)⁺ RNA. Total p(A)⁺ egg RNA was denatured at 70 °C in 67% formamide, 0.3 M NaCl, 0.01 M PIPES (pH 6.5), 10⁻⁴ M EDTA for 1 min, then incubated at 30 °C in 58% formamide, 0.75 M NaCl, 0.025 M PIPES (pH 6.5), 10⁻⁴ M EDTA, to equivalent C_0t 600 M s. The RNA was then diluted and spread for electron microscopy⁴¹ from a hyperphase of 80% formamide, 0.1 M Tris (pH 8), 5 mM EDTA and 100 $\mu\text{g ml}^{-1}$ cytochrome c, at 24 °C. Many multimolecular RNA structures, including large networks, were observed. The montage shown includes several relatively simple examples of such structures. The bar represents a single-strand RNA length of 1,000 nucleotides, estimated from circular single-stranded ΦX174 DNA included on the same grids, and including a 10% correction for the difference in linear densities of single-stranded RNA and DNA in these spreading conditions⁴². To estimate the fraction of RNA in multimolecular structures, random areas of the grid were photographed, and the total contour length of each structure was measured. The structures measured had a total contour length of 1.2×10^6 nucleotides, of which 65% was contained in structures having four or more discernible ends and judged to consist of two or more RNA molecules. Sea urchin rRNA did not form networks when incubated to the same C_0t and spread in identical conditions. When the p(A)⁺ RNA was spread in the same conditions following denaturation ($C_0t < 2 \times 10^{-3}$ M s), no networks were observed, and less than 4% of the RNA length was in structures containing possible intermolecular duplexes. The denatured p(A)⁺ RNA had a weight-average length of 5,000 nucleotides, and a number-average length of 3,400 nucleotides, based on a sample of 187 randomly selected molecules. Size determinations by agarose gel electrophoresis in the presence of methyl mercuric hydroxide⁴³ were consistent with these values.

molecules. It follows that many p(A)⁺ RNA molecules bear more than one repeat sequence element. Otherwise, almost all renatured structures would include only two molecules and very large networks such as some of those shown in Fig. 2 would never have formed. Another general implication is that most of the length of those p(A)⁺ RNA molecules which contain repetitive sequence elements consists of single-copy sequence transcript. This is demonstrated directly in the next section.

Interspersion of repetitive and single-copy sequences in egg p(A)⁺ RNA

To examine the sequence organization of p(A)⁺ RNA molecules containing repeat transcripts, it was necessary to purify these molecules with respect to the remainder of the RNA. The following strategy proved effective. Egg p(A)⁺ RNA was labelled *in vitro* with ¹²⁵I (refs. 19–21), and hybridized with excess repetitive DNA which had previously been mercurated (Hg-DNA)²². Details are given in the legend to Fig. 3. Excess non-mercurated sea urchin ribosomal DNA (rDNA) was added to compete out the hybridization of contaminating ¹²⁵I-rRNA, which amounted to 1–5% of the various ¹²⁵I-p(A)⁺ RNA preparations. After denaturation, the reaction mixtures were incubated to C_0t 50 M s with respect to the repetitive Hg-DNA, and fractionated on a sulfhydryl-agarose (SH-agarose) column. RNA molecules hybridized to the repetitive DNA are bound to this column, while non-hybridized RNA flows through. When mixed with the Hg-DNA (denatured or native) but not permitted to anneal, less than 1% of the ¹²⁵I-p(A)⁺ RNA bound to the SH-agarose column. This background is somewhat lower than is obtained with the urea-phosphate hydroxyapatite-binding procedure²³ for trapping RNA hybrids formed with excess DNA. Control experiments showed that the same amount of

hybrid was recovered with the SH-agarose Hg-DNA procedure, as by the urea-phosphate hydroxyapatite-binding method.

The kinetics of the hybridization reaction between the ¹²⁵I-p(A)⁺ RNA and the repetitive Hg-DNA are illustrated in Fig. 3. To provide relative kinetic standards, Fig. 3 includes reactions of a genomic ³H-DNA repeat tracer and of a single-copy ³²P-DNA tracer with the same Hg-DNA driver. The reaction of the ¹²⁵I-p(A)⁺ RNA occurs at approximately the same rate as the reaction of the repeat DNA tracer. As expected, single-copy sequences are more than 10-fold reduced in concentration in the repetitive Hg-DNA driver compared to whole DNA. There is no reaction whatsoever of the ³²P-DNA single-copy tracer until $C_0t > 10^2$. Therefore, the ¹²⁵I-p(A)⁺ RNA molecules which hybridize with the Hg-DNA at C_0t 10⁻²– C_0t 10² M s (Fig. 3) must contain repeat sequences. The kinetics exclude the possibility that the trapped RNA molecules were bound by virtue of hybridization with residual single-copy sequences contaminating the repetitive Hg-DNA driver. Furthermore, when the bound RNA is eluted and again reacted with the repetitive Hg-DNA driver, the kinetics observed are consistent with those in the initial reaction.

Although binding the p(A)⁺ RNA–Hg-DNA hybrids to SH-agarose effectively isolates molecules which include repeat transcripts, the yield of the procedure is low. After hybridization, only 16–19% of the ¹²⁵I-p(A)⁺ RNA binds to the SH-agarose column. In the presence of excess sea urchin rDNA, the fraction of ¹²⁵I-RNA bound falls slightly, to 11–16%. Several factors suggest that this value seriously underestimates the true fraction of p(A)⁺ RNA molecules which include repeat-sequence elements: (1) The repetitive DNA driver was prepared by incubation of the starting DNA to C_0t 50 M s resulting in under-representation of repeat sequences whose frequency of occurrence is less than 10–20 copies per genome. (2) The

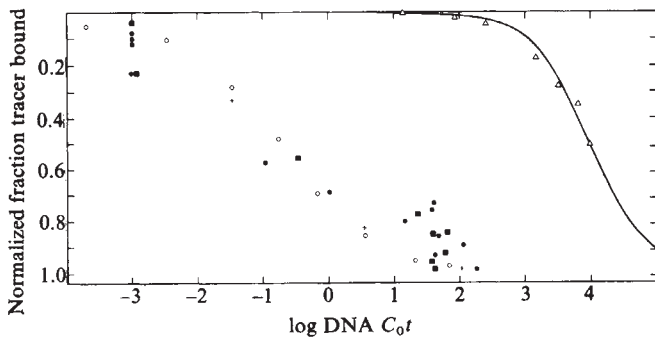


Fig. 3 Kinetics of hybridization of $^{125}\text{I-p(A)}^+$ RNA with excess mercurated repetitive DNA. $^{125}\text{I-p(A)}^+$ egg RNA was hybridized with a 100- to 1,000-fold excess of mercurated repetitive sea urchin DNA and hybridization was assayed by binding to SH-agarose. To facilitate kinetic comparisons, hybridization values are normalized to the maximal extent of reaction in each curve. All reaction mixtures were denatured in 65–75% formamide, 0.3 M NaCl at 75–80°C, then incubated in 50% formamide, 0.75 M NaCl, 0.025 M PIPES (pH 6.5), 10^{-4} M EDTA, 0.1% SDS and 5×10^{-5} M NaCN, at 30°C. Binding to SH-agarose⁴⁴ was performed in 0.5 M NaCl, 0.01 M Tris (pH 7.5), 10^{-3} M EDTA, 0.05% SDS, and the bound nucleic acids were eluted with the same buffer containing 0.1 M β -mercaptoethanol. Repetitive sea urchin DNA was prepared by S_1 digestion of total DNA after reassociation to C_0t 50 M s (refs 45 and 46), and the repetitive DNA was covalently mercurated²². 85–90% of native or denatured mercurated DNA could be bound to SH-agarose. The abscissa indicates equivalent C_0t values⁴⁵, including an estimated 2.5-fold rate retardation due to hybridization in 50% formamide⁴⁷. ■, hybridization of $^{125}\text{I-p(A)}^+$ RNA in the presence of excess non-mercurated rDNA competitor (normalized from a 13% terminal value); ●, the same, but without rDNA competitor (normalized from a 20% terminal value); +, re-reaction of hybridized $^{125}\text{I-p(A)}^+$ RNA with more repetitive Hg-DNA driver, plus rDNA competitor (normalized from a 35% terminal value). For comparison the hybridization kinetics of short repetitive $^3\text{H-DNA}$ (○) (normalized from a 90% terminal value), and single-copy $^{32}\text{P-DNA}$ tracer (Δ) (normalized from a 95% terminal value obtained with unfractionated DNA driver) are shown, with the same repetitive Hg-DNA driver. The latter two reactions were assayed by binding to hydroxyapatite.

reaction criterion (50% formamide, 0.75 M NaCl at 30°C) probably precludes duplex formation within the less closely related classes of repeat sequences found in the sea urchin genome²⁴. (3) The p(A)^+ RNA was unavoidably subjected to some strand scission during iodination. The weight-average length of the total iodinated p(A)^+ RNA was about 1,400 nucleotides, as measured on denaturing formaldehyde–sucrose gradients²⁵. $^{125}\text{I-p(A)}^+$ RNA which had been hybridized with Hg-DNA, bound to SH-agarose, and eluted displayed the same length distribution. While the hybridization procedures thus result in no further strand scission, the fragment length of the p(A)^+ RNA after iodination is less than one-third its starting length. Unless all repeat sequences in the RNA are distributed less than 1,400 nucleotides apart, this amount of breakage would have resulted in a decrease in the fraction of p(A)^+ RNA bound after hybridization with the repetitive Hg-DNA. (4) The SH-agarose column binds only about 40% of the available repetitive RNA–DNA duplexes. As shown in Table 1, when RNA that fails to bind after reaction with Hg-DNA is re-hybridized, another 8–13% can be bound. When once-bound RNA molecules, all of which must contain a hybridizable repeat sequence, are eluted and re-hybridized, only 40% bind to a second SH-agarose column. A probable explanation, according to experiments not shown here, is that duplexes between mercurated and non-mercurated nucleic acids bind with less than 50% efficiency to SH-agarose. This phenomenon was also reported earlier by Nguyen-Huu *et al.*²⁶. Using the data of Table 1, we estimate that about 35% of 1,400 nucleotide p(A)^+ RNA fragments actually contained recognizable RNA–DNA hybrids (that is, 15% initial binding normalized for a 40% SH-agarose binding efficiency). This estimate is still two-fold below that obtained from our electron microscopic observations. The most likely explanation is the difference of over threefold in RNA fragment length in the two experiments. In addition, more repeat duplexes may have survived the conditions of the elec-

tron microscope experiment than could be recovered as Hg-DNA–RNA hybrids, due to the relatively high stability of RNA–RNA duplexes in formamide media.

The repetitive and single-copy sequence content was measured in a sample of $^{125}\text{I-p(A)}^+$ RNA which had been twice reacted with the repeat Hg-DNA driver, in the presence of rDNA competitor, and bound to SH-agarose (preparative data are shown in Table 1). After elution, the RNA was hybridized with excess whole sea urchin DNA, and the ribonuclease resistance of the $^{125}\text{I-RNA}$ was determined as a function of DNA C_0t (refs 23, 27). Data are shown in Fig. 4. Least-squares analysis of this reaction indicates that 85–90% of the mass of the selected $^{125}\text{I-RNA}$ is single-copy sequence transcript. However, these single-copy sequences must be linked to repetitive sequences, since every RNA molecule in the selected $^{125}\text{I-RNA}$ fraction contains at least one repeat-sequence element.

The repetitive sequences in the bound p(A)^+ RNA are generally short, although a small fraction of the RNA could consist entirely of repeat transcripts. Figure 4 suggests that the average 1,400-nucleotide $^{125}\text{I-RNA}$ fragment bound to SH-agarose includes less than 200 nucleotides of repetitive sequence. In other experiments $^{125}\text{I-p(A)}^+$ RNA was reacted with the Hg-DNA, bound to SH-agarose and the hybrid molecules were eluted and digested directly with ribonuclease. Only 8–12% of the $^{125}\text{I-RNA}$ was resistant, again indicating that there are no more than 170 nucleotides of repeat sequence on the average RNA fragment in this fraction. It is unlikely that a significant amount of hybridized repetitive RNA was rendered soluble in trichloroacetic acid (TCA) by the ribonuclease digestion since the amount of resistant RNA was the same when the assay was carried out at 0°C as at 37°C. It is nonetheless

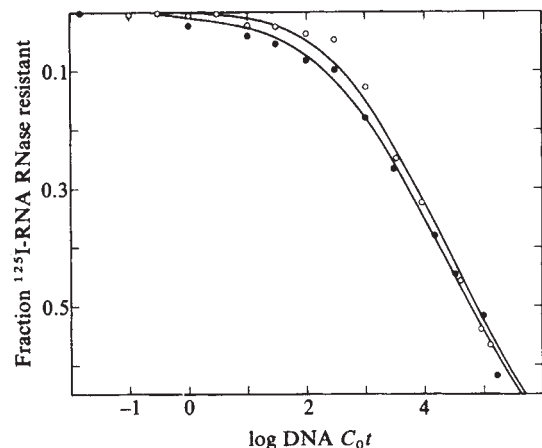


Fig. 4 Repetitive and single-copy sequence content of repeat-containing egg p(A)^+ RNA. A fraction of p(A)^+ RNA, consisting of transcripts containing repeated sequences, was selected by hybridization with repetitive Hg-DNA and chromatography on SH-agarose (see text). Hybridization of contaminating $^{125}\text{I-ribosomal RNA}$ was blocked by competition with non-mercurated cloned sea urchin ribosomal DNA. This selection procedure was then repeated to assure purity (see Table 1). The final selected $^{125}\text{I-RNA}$ (●), or total $^{125}\text{I-p(A)}^+$ egg RNA (○), was then reacted with a 10^5 – 10^6 -fold excess of total sea urchin DNA. Hybridization was assayed by digestion with $10 \mu\text{g ml}^{-1}$ ribonuclease A for 1 h at 37°C in 0.24 M phosphate buffer (pH 6.8), 10^{-3} M EDTA, followed by precipitation with cold 10% TCA and collection on Whatman GF/C filters. Hybridized RNA sequences are resistant to digestion in these conditions, while single-strand regions are hydrolysed. To estimate the fractions of $^{125}\text{I-RNA}$ hybridizing with repetitive or with single-copy sequences in the driver DNA the data were fitted using a function that takes into account the retarded hybridization of RNA tracers⁴⁸, as well as the effects of a nuclease-resistance assay on the observed kinetics^{49–51}. The fraction of the selected $^{125}\text{I-RNA}$ hybridizing as a repetitive component appears to be between 10% and 15%, depending on the allowed average repetition frequency. A reasonable estimate, assuming an average frequency of 100 copies per genome, suggests that 89% of the selected RNA fraction consists of single-copy sequence transcript, and 11% of repeated-sequence transcript, while the total p(A)^+ RNA is 93% single-copy sequence transcript and 7% repeated-sequence transcript. These solutions are indicated by the solid lines shown. The total $^{125}\text{I-p(A)}^+$ RNA preparation contained about 1% ribosomal RNA measured by hybridization with cloned ribosomal DNA.

Table 1 Fraction of ^{125}I -p(A)⁺ RNA binding to SH-agarose after hybridization with excess mercurated repetitive DNA

Experiment	First reaction + rDNA	% bound		Re-reaction of unbound fraction + rDNA
		Re-reaction of bound fraction + rDNA	- rDNA	
1	15	42	40	8
2	16	30,35	40	13

Hybridization and assay conditions are described in the legend to Fig. 3. All hybridizations were carried out at DNA C_0t 50 M s. In each experiment, egg ^{125}I -p(A)⁺ RNA was hybridized with a 100-fold mass excess of mercurated repetitive DNA, in the presence of a large sequence excess of non-mercurated cloned sea urchin rDNA³⁹. The reaction mixtures were then chromatographed on an SH-agarose column. Aliquots of the bound fraction were denatured and re-reacted with additional mercurated repetitive DNA, plus or minus rDNA competitor, as indicated. The unbound fraction was similarly re-reacted in the presence of rDNA competitor.

possible that the average length of the repeat sequences could be somewhat greater than 170–200 nucleotides. In any case we conclude that at least 35% (and perhaps twice this fraction) of sea urchin egg p(A)⁺ RNA has an interspersed sequence organization, in which short repetitive sequences are covalently associated with longer single-copy sequence transcripts.

Complexity of single-copy sequences linked to repeats in egg RNA

In this section, we demonstrate that the interspersed egg RNAs include most of the diverse species of single-copy sequence transcript found in the egg, and therefore, most of the diverse maternal mRNAs. To measure the complexity of the single-copy sequence transcripts associated with repeats in the egg RNA, we prepared a selected fraction of total egg RNA in which the interspersed RNA molecules were relatively concentrated. We then hybridized this fraction with an eDNA tracer. Hough-Evans *et al.*¹² showed earlier that at least 75% of the single copy sequence represented in total egg RNA is included in the mRNA of early embryo polysomes. The eDNA tracer is therefore a probe for the complex assemblage of single-copy sequences comprising the maternal message set stored in the sea urchin egg.

The RNA fraction required for this complexity measurement was obtained by hybridizing total egg RNA with repetitive Hg-DNA, followed by chromatography on SH-agarose. The egg RNA/Hg-DNA mass ratio was sufficient to permit hybridization of most of the diverse repeat transcripts, but very little of the egg rRNAs or tRNAs (see legend to Fig. 5). After hybridization, 2.2% of the total egg RNA bound to the SH-agarose column. The Hg-DNA was eliminated by DNase treatment, and a measurement was made of the enrichment for repeated sequence transcripts actually obtained in the fractionation. Thus, the concentration of transcripts complementary to two cloned repetitive sequences was determined by titration with the selected RNA fraction. Figure 5 shows that transcripts complementary to the repeat sequence of clone CS2109B are present at an 11-fold higher concentration in this RNA fraction than in total egg RNA. Similarly, the repeat sequence of clone CS2111 (ref. 24) is nine times more concentrated in the selected RNA fraction. We expect, therefore, that single-copy sequence transcripts linked to repeats will also be concentrated in the selected RNA fraction by a factor of about 10, with respect to total egg RNA.

The kinetics of reactions between a ^{32}P -eDNA tracer and the selected RNA fraction, as well as the reaction of the ^{32}P -eDNA with total egg RNA, are shown in Fig. 6a. This tracer contained no detectable repetitive-sequence component (data not shown). Both of the eDNA hybridization reactions in Fig. 6a terminate at about 85% of the tracer bound. Therefore, the selected RNA fraction includes essentially all the diverse single-copy sequences in total egg RNA. The experiment clearly shows that the single-copy sequence transcripts driving the eDNA reaction are concentrated in the selected RNA fraction. Least-squares solutions to the kinetic data indicate that 70–80% of the reac-

tion is accelerated 10-fold compared to total egg RNA, while the remaining 20–30% is accelerated 2-fold. It follows that at least 70–80% of the diverse single-copy sequences in egg RNA exist on transcripts also containing repeated sequences. Since the large majority of the same single-copy sequences are represented in polysomal mRNA after fertilization, most, if not all, of the interspersed egg RNAs are maternal messages. Furthermore, interspersed transcripts are the major form of these maternal mRNA species, rather than a minor variant. If, for example, only 25% of the transcripts of a particular single-copy sequence contained a repeat sequence, a 10-fold enrichment for that repeat would result in only a 2.5-fold enrichment for the single-copy sequence. It is possible that the 20–30% of single-copy sequences that appear to be less enriched in the selected egg RNA fraction reside on transcripts which lack repeats. Alternatively, these sequences might simply represent regions of transcripts that are relatively distant from repeats and are often separated from them by a strand scission.

Interspersed repetitive sequences exist in prevalent as well as rare or complex-class maternal messages. This was shown by titration experiments with a cloned cDNA fragment, SpG30, which represents a highly prevalent egg p(A)⁺ mRNA. There are about 2×10^5 molecules of this RNA per egg. The cloned SpG30 fragment is about 700 nucleotides long and consists entirely of single-copy sequence¹³. Figure 6b illustrates titration measurements of the sequence concentration of transcripts complementary to the strand separated SpG30 probe in total egg RNA, and in the repeat-enriched selected RNA fraction. Like most rare single-copy transcripts, this highly prevalent single-copy sequence is also about 10-fold more concentrated in the repeat-enriched RNA fraction. A repetitive sequence therefore must be located somewhere on the SpG30 transcript.

Discussion

We have shown that a substantial fraction of the heterogeneous RNA in the sea urchin egg, including the p(A)⁺ RNA, consists of short repetitive sequence transcripts covalently linked to longer single-copy sequences. While these interspersed transcripts have many properties that identify them as maternal messages, we have not demonstrated that they actually function as messages. However, most of the diverse message sequences in the egg are found on molecules which display an interspersed sequence organization. In addition, essentially all of the mass of the heterogeneous repeat-sequence transcripts in the egg⁵ can

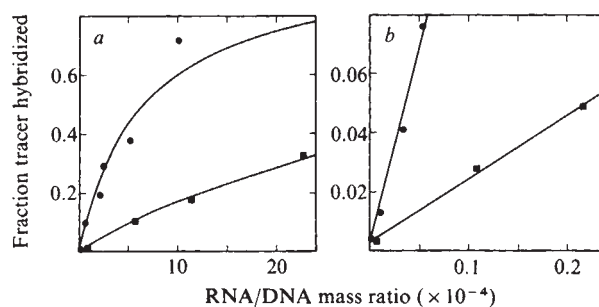


Fig. 5 Concentration of repetitive sequence transcripts in a selected fraction of egg RNA. To yield a fraction enriched for transcripts containing repeated sequences, 1 mg of unlabelled total egg RNA plus 8 μg of ^{125}I -labelled total egg RNA was hybridized with 1 mg of repetitive Hg-DNA. This is a sufficient DNA/RNA ratio to provide a DNA sequence excess for any repeat present in more than 20 copies per genome and contained on fewer than 2×10^5 transcripts per egg. The reaction mixture was incubated to Hg-DNA C_0t 50 M s, then fractionated on an SH-agarose column, as described in Fig. 3. The bound fraction was eluted with 0.1 M β -mercaptoethanol, and the repetitive Hg-DNA was completely removed by DNase digestion. Two ^{32}P -labelled cloned repetitive sequences were then titrated with total egg RNA and with the selected RNA fraction. *a*, Titration of the repeat sequences from clone CS2109B, lower strand (■), and clone CS2111, lower strand (●) with total egg RNA. *b*, Titration of the same two repeat sequences with the selected repeat-enriched RNA. The complementary RNA fractions are: CS2109B, 2.1×10^{-6} of total egg RNA and 2.3×10^{-5} of repeat-enriched RNA; CS2111, 1.5×10^{-5} of total egg RNA and 1.4×10^{-4} of repeat-enriched RNA.

be accounted for as interspersed RNA molecules. The precise physical organization of these maternal RNAs is not yet apparent. This will require further analysis of individual transcripts using cloned DNA probes. We know, however, that the repetitive sequence elements are relatively short, averaging only 150–200 nucleotides. According to primary sequence data, six out of eight cloned repeat elements contain translation stop codons in all reading frames³, and this observation suggests that the repeats may be located elsewhere than in translated regions of the message. It is also likely that more than one repeat-sequence element exists on many RNA molecules. A possibility not yet excluded is that the repeat-sequence elements are removed by cytoplasmic processing events before polysomal translation. It is also possible, if unlikely, that only the minor fraction of egg transcripts which lack repeats serve as maternal messages.

Is interspersed sequence organization a feature peculiar to sea urchin maternal RNAs? Earlier studies on the newly synthesized polysomal mRNA of gastrula stage sea urchin embryos failed to reveal a significant fraction of message containing linked repetitive and single-copy sequences^{23,28}. There have been several reports on the sequence organization of messages from other animal cell types (see, for example, refs 29–32). With the aid of cloned probes, Firtel and his associates recently clearly demonstrated that short repeat-sequence elements exist on certain predominantly single copy *Dictyostelium* mRNAs^{33,34}. Re-examination of sea urchin gastrula mRNA by the methods described here, as well as those previously used reveals a small fraction of polysomal mRNAs which contain interspersed repeats (unpublished data). However, a major distinction exists in the amount of repetitive-sequence transcript in egg maternal RNA as opposed to gastrula polysomal mRNA. While the egg contains many highly prevalent repeat transcripts⁵, the gastrula mRNA seems to contain few if any repeat-sequence transcripts of equally high prevalence. The only highly prevalent interspersed repeat transcripts observable in the sea urchin gastrula are found in the nucleus, as concluded earlier by

Scheller *et al.*⁶. The process by which this intracellular distribution of repeat transcripts is achieved during development is unknown, as is its functional significance.

Our most important conclusion pertains to the sequence organization of the sea urchin genome and its possible function. We found earlier²⁸ that the single-copy sequences represented in embryo mRNA tend to be located non-randomly, close to interspersed repetitive sequences in the genome. Our present data require the further conclusion that most of the structural genes represented in egg RNA belong to sets, each of which is defined by the presence of homologous sequences of a given repeat family. This can be seen as follows: According to previous measurements, transcripts of 10–20% of the diverse repetitive sequence families in the genome, or 500–1,000 different sequence families, are prevalent in the egg RNA⁵. About 10^5 transcripts represent each of these prevalent repetitive sequence families per egg, and together these account for $\geq 90\%$ of the mass of interspersed repeat transcripts. No more than a few per cent of the mass of the repeat transcripts are accounted for by rare repeat sequences, that is, those represented in only 10^3 – 10^4 copies per egg⁵. On the other hand, 60–80% of the maternal mRNA in the egg consists of rare, complex-class single-copy sequence transcripts (refs 7, 12 and unpublished data). Most of these complex-class transcripts are covalently linked to repeats, according to the experiment of Fig. 6a, and most of the repeats must belong to the prevalent repeat-transcript class⁵. Since the complexity of the egg single-copy sequence set is 3.7×10^7 nucleotides^{12,35}, whereas there are only a few hundred kinds of prevalent repeat transcripts, a majority of these repetitive-sequence families must be represented on many (probably between 10 and 50) different rare messages. It follows that the prevalent repeat transcripts belonging to each family must have derived from many distinct transcription units, each including a different single-copy gene (or a few such genes). Thus, both the structural genes expressed during oogenesis and the many thousands of mRNA species produced by their transcription are distributed among several hundred sets, according to the particular repetitive-sequence family represented on each mRNA molecule. A similar organization has been observed in *Dictyostelium*^{33,34}, where two prevalent repeat transcripts each appear to be associated with 50–100 different rare mRNAs.

The organization of structural genes in sets or 'gene batteries', each associated with a specific repeat family (or families), has been proposed in models for regulation of gene expression^{36,37}. It was envisioned that the shared repeats of each battery provide the physical basis for coordinate control of its expression. It is easy to see that the distribution of only 10–20% of the different repeat sequences in the genome among the more than 10^4 single copy message species in the egg implies a non-random arrangement of certain repetitive sequences among those genes which are expressed during oogenesis. However, there is no evidence regarding the actual function, if any, of the transcribed interspersed repeats. Their presence in the maternal RNA provides an unexpected opportunity for further examination of the physiological meaning of genomic sequence interspersion. Among the functions which might be envisioned for these repeat transcripts are suppression of mRNA utilization before fertilization, translational control affecting the timing of utilization of specific mRNA sets following fertilization, control of the differential distribution of maternal mRNAs to various regions of the early embryo, or sites for specific RNA processing events before translation. Alternatively, they might be remnants of transcriptional or post-transcriptional regulatory signals^{36,37}, incompletely removed from germinal vesicle precursors. A different possibility is that some of the maternal repeat transcripts could serve in a regulatory capacity in early embryo nuclei, where they might be involved in imposing initial patterns of gene expression which are similar to those in effect during oogenesis^{12,35,38}.

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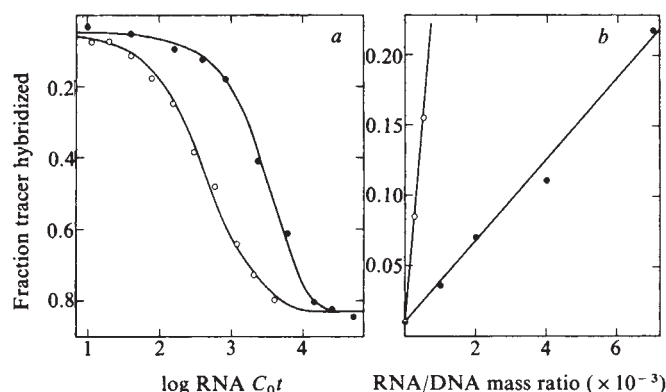


Fig. 6 Complexity of single-copy sequence transcripts included in the selected repeat-enriched RNA fraction. *a*, Hybridization of an eDNA ³²P-labelled tracer containing all the single-copy sequences represented in total egg transcripts with excess total egg RNA (●) or with the selected repeat-enriched egg RNA fraction (○) described in Fig. 5. The ³²P-labelled eDNA tracer was prepared, as previously described¹², by hybridizing total single copy ³²P-DNA with total egg RNA and purifying the hybridized fraction. The eDNA preparation used in this experiment was about 30-fold enriched for egg RNA sequences relative to the starting single-copy ³²P-DNA preparation. Hybridization was dependent on added RNA, and hybrids were sensitive to RNase digestion in low salt⁵². The curve shown for the total egg RNA reaction describes a single pseudo-first-order kinetic component with a rate constant of $2.3 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$. In the kinetic solution shown here for the reaction with the repeat-enriched RNA fraction two components were assumed. The rate constant for the faster component was fixed at $2.3 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ or 10 times the rate constant obtained with total egg RNA so as to estimate the fraction of single-copy sequences enriched to the same extent as repeats. This component included 70% of the reaction, while 30% displays a rate constant of $4.1 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$. *b*, Titration of the ³²P-labelled single-copy DNA sequence of clone SpG30, upper strand, with total egg RNA (●) or with the repeat-enriched egg RNA fraction (○). The complementary fraction of total egg RNA was 3.0×10^{-5} , while the complementary fraction of repeat-enriched RNA was 2.9×10^{-4} .

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Note added in proof: The characteristics of sea urchin egg p(A)⁺ RNA described here pertain as well to the p(A)⁺ RNA of *Xenopus*, an anuran vertebrate. When p(A)⁺ RNA from the unfertilized egg of *Xenopus* (obtained by Dr L. Dennis Smith) was renatured and spread for electron microscopy under the exact conditions of Fig. 2 (by M. E. Chamberlin), the following results were obtained. (1) RNA which was not permitted to anneal after denaturation displayed only occasional intrastrand foldback structures. (2) RNA renatured to C₀t 60 was largely involved in structures containing two to four molecules bound by short duplex regions. And (3), RNA renatured to C₀t 600 was largely included in complex multimolecular aggregates often containing more than 10 molecules.

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Structure of a family of rat amylase genes

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The sequences of two cloned rat pancreatic amylase cDNAs comprising 95% of the mRNA sequence are reported. Analysis of cloned rat genomic DNA fragments using cloned cDNA probes indicates that the rat genome contains multiple closely related amylase genes in which the cDNA sequences are distributed within a region 9 kilobases in length and are interrupted by at least seven intervening sequences.

α -AMYLASE confers on the cell or organism producing it the capacity to use polyglucosides with α -1,4 linkages (such as starch) as energy and carbon sources. The rat genome, like other mammalian genomes, contains multiple closely related amylase genes which are selectively expressed in a number of differentiated cells. Amylase is a major product of the pancreas and parotid glands, accounting for approximately 20% of the total protein^{1,2}, and is associated with the digestive functions of these tissues. Liver also produces amylase but in relatively low quantities³; it is a prominent source of serum amylase in the normal animal³.

Because of its prominent functional role amylase has served as a specific marker of differentiation⁴⁻⁹. The synthesis of amylase is initiated in parotid and pancreas cells at different periods of embryological development according to the differentiative

programmes of these cells¹⁰. The synthesis of amylase in pancreatic acinar cells is apparently dependent on insulin¹¹, and can be induced fourfold by glucocorticoids so that it represents more than 70% of the total protein synthesis¹². The difference in amylase levels in pancreas, parotid and liver parallel the relative abundance of amylase mRNA in the tissue¹³. Thus it appears that amylase synthesis is largely regulated at the level of mRNA synthesis or degradation.

The results of a variety of studies suggest the amylases of pancreas, parotid and liver, though similar, are distinct proteins and are the products of at least three distinct genes. The amylases isolated from the pancreas and parotid are immunologically distinguishable and have clearly different peptide maps^{14,15}. Immunological studies also suggest differences in the parotid and liver enzymes¹⁶. The melting profiles of complementary (c)DNA-RNA hybrids formed with pancreatic amylase cDNA indicate greater than 90% sequence homology between pancreatic, parotid and liver amylase mRNAs but also clearly distinguish the pancreatic mRNA from the parotid and liver mRNAs¹³. Preliminary evidence suggests differences between parotid and liver amylase mRNAs as well¹³.

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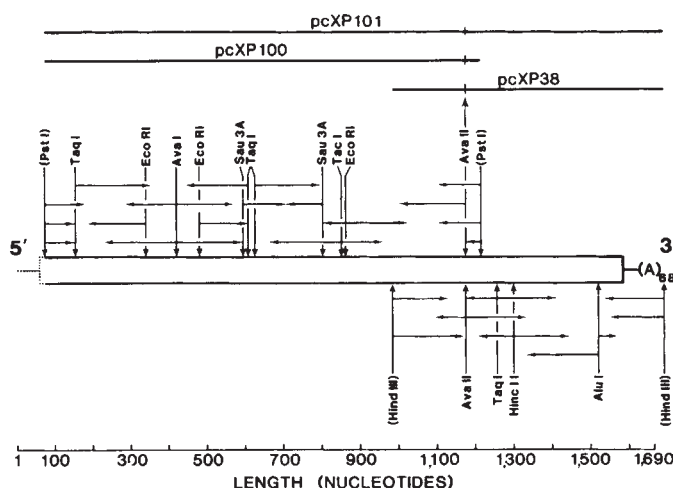


Fig. 1 Sequencing strategy for rat pancreatic amylase mRNA. The extent of the two double-stranded cDNA cloned sequences is discussed in the text. The direction of each sequencing reaction is shown by the horizontal arrows, starting at the restriction sites shown. (The number of bases determined in each reaction is indicated by the length of the arrows.) The 5' end-labelled restriction fragments for sequencing were prepared from double-stranded cDNA separated from the pBR322 sequences. The internal *HindIII* site was derived from the *HindIII* restriction site linker added to the pcXP38 insert during the double-stranded cDNA cloning. The dotted line at the 5' end indicates the uncloned portion of the mRNA.

In view of their common ancestral origin and their selective expression in differentiated cells, the amylase genes present an unusually favourable opportunity for analysis of structure-function relationships in eukaryotic genes. We describe here the nucleotide sequence of amylase mRNA derived from a nearly full-length cDNA cloned in bacteria. From this nucleotide sequence we have deduced the amino acid sequence of pancreatic amylase and certain characteristics of the amylase molecule.

Using the cDNAs as probes we have isolated genomic DNA fragments containing amylase genes. We present here the overall structure of the several amylase genes present in the rat genome.

Sequence of amylase cDNA

The details of molecular cloning of the population of predominant rat pancreatic mRNAs as cDNA and the identification of cloned sequences for a number of secretory enzyme mRNAs including amylase will be described in detail elsewhere¹⁷. Approximately 220 recombinant clones containing pancreatic amylase cDNA sequences have been identified among approximately 1,400 recombinant bacterial transformants. Restriction endonuclease analysis revealed two overlapping cDNA clones, designated pcXP38 and pcXP100, which contain cDNA inserts of 702 and 1,176 base pairs, respectively. These were sequenced by the method of Maxam and Gilbert¹⁸, according to the sequencing strategy shown in Fig. 1. The sequence is given in Fig. 2. The pcXP38 cDNA insert contains a terminal poly(A) stretch of 68 residues and therefore encodes the 3' end of the mRNA. The pcXP100 insert overlaps with 227 nucleotides at the 5' end of the pcXP38 insert (Fig. 1); thus the total length of the mRNA sequence contained in the two plasmids is 1,615 nucleotides. The length of pancreatic amylase mRNA, estimated by agarose gel electrophoresis in the presence of methyl mercury¹⁹, is 1,690 $\pm$ 30 nucleotides¹⁷ (average of 12 determinations) including an average poly(A) length of 65 nucleotides⁸. The sequence contained in the two cloned cDNAs therefore represents greater than 95% of the total mRNA sequence and lacks 75 $\pm$ 30 nucleotides at the 5' end of the mRNA.

The nucleotide sequence of the 227-base pair overlap common to both cloned cDNAs is identical at all positions except

position 1,139 (Fig. 2) which is unambiguously T in pcXP38 and A in pcXP100. This discrepancy may have been introduced by the cloning procedures²⁰, or may reflect the cloning of two closely related, perhaps allelic, pancreatic amylase mRNAs.

Organization of the amylase mRNA sequence

The amino acid sequence of rat pancreatic amylase, derived solely from the nucleotide sequence of the mRNA, is given in Fig. 2. The amino acid sequence of the protein has not yet been determined, so comparison of independent amino acid and nucleotide sequences is not possible; however, the authenticity of the proposed reading frame and the consequent amino acid sequence is supported by the following criteria: (1) the reading frame shown is the only one without multiple termination codons; (2) the calculated molecular weight of the proposed amylase sequence, including amino-terminal residues, which probably correspond to a portion of the prepeptide, is 56,570. This is similar to the 58,000 molecular weight estimate for the rat pancreatic amylase precursor synthesized *in vitro*⁵; (3) the amino acid composition of the deduced sequence coincides with that reported for rat pancreatic amylase^{17,21}. The fractions of aspartate plus asparagine (17%) and tryptophan (3.5%) are characteristically high for amylase²¹ compared with the average for 314 proteins compiled by Dayhoff *et al.*²². The amino acid compositions of the multiple potential polypeptides predicted by each of the other two reading frames are dissimilar; (4) digestion of purified rat pancreatic amylase with phenylmethylsulphonyl fluoride (PMSF) treated carboxypeptidase A in the conditions described by Ambler²³ releases only leucine (data not shown), consistent with the carboxyl terminal dipeptide, Lys-Leu, predicted by the proposed coding sequence²⁴; (5) the amino acid sequence is 76% homologous with the amino acid sequence of a number of unordered peptides that account for approximately 75% of the total sequence of porcine pancreatic amylase (provided by L. Paseno, B. Alandie, Y. Mazzei, D. Moinier, J. P. Bizzozero, M. Fougereau, Y. Chickeportiche and G. Marchis-Mouren). It is significant that porcine pancreatic amylase has a lower molecular weight than the rat enzyme by 3,000–5,000 (ref. 25) and has an isoelectric point of 5.4 (ref. 25), compared with 8.8–9.0 for rat pancreatic amylase¹⁵. Despite these differences the sequence homology between the two enzymes is high.

The amino-terminal portion of the predicted amino acid sequence for rat pancreatic amylase (Fig. 2) can be uniquely aligned with the partial amino-terminal sequence of rat parotid preamylase²⁶ so that seven of the nine identified residues in a region 27 residues long are congruent. This alignment suggests that the cDNA sequence lacks four-and-one-third codons or 13 coding nucleotides including the initial AUG but contains the carboxyl-terminal portion of the prepeptide. The entire coding region of rat pancreatic amylase mRNA is thus probably 1,551 nucleotides long, encoding 517 amino acids. The exact carboxyl terminus of the prepeptide, that is, the point of cleavage by a signal peptidase²⁷, cannot be located at this time because the amino terminus of the mature enzyme is acetylated and consequently has not been identified.

The single nucleotide discrepancy within the overlapping sequences of pcXP38 and pcXP100 (position 1,139) is silent with respect to the amino acid sequence since both of the alternative codons, AUA and AUU, specify Ile 379.

The length of the 5'-untranslated region, estimated to be 60 $\pm$ 30 nucleotides, is within the range of other reported 5'-untranslated regions, that is, from 32 nucleotides in mouse β -globin mRNA²⁸ to greater than 112 nucleotides in the ACTH β -lipotropin mRNA²⁹. In contrast, pancreatic amylase mRNA has a very short 3'-untranslated region relative to other eukaryotic mRNAs which have been characterized. The 3'-untranslated region of pancreatic amylase mRNA is only 35 nucleotides long whereas the reported lengths of 3'-untranslated regions in other mRNAs range from 57 nucleotides for rat insulin³⁰ mRNA to 950 nucleotides for mouse dihydrofolate reductase mRNA³¹. A short 3'-untranslated region is not a


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1      10      20      30      40      50      60      70      80      90      100      110      120      130      140      150      160      170      180      190      200      210      220      230      240      250      260      270      280      290      300      310      320      330      340      350      360      370      380      390      400      410      420      430
leu leu ser leu ile gly phe cys trp ala gln tyr asp pro his
UG CUG CUU UCC CUC AUU GGG UUC UGC UGG GCU CAA UAU GAC CCA CAC

thr ala asp gly arg thr ala ile val his leu phe glu trp arg trp
ACU GCG GAU GGG AGG ACU GCU AUU GUC CAC CUG UUC GAG UGG CGC UGG

ala asp ile ala lys glu cys glu arg tyr leu ala pro lys gly phe
GCU GAU AUU GCC AAG GAA UGU GAG CGG UAC UUA GCA CCU AAG GGA UUU

gly gly val gln val ser pro asn glu asn ile ile ile asn asn
GGA GGG GUG CAG GUC UCU CCA CCC AAU GAA AAU AUU AUA AUU AAU AAU

pro ser arg pro trp trp glu arg tyr gln pro ile ser tyr lys ile
CCA UCA AGG CCU UGG UGG GAA AGA UAU CAA CCA AUC AGC UAC AAA AUU

cys ser arg ser gly asn glu asn glu phe lys asp met val thr arg
UGC UCA AGG UCU GGA AAU GAA AAU GAA UUC AAA GAC AUG GUG ACG AGG

cys asn asn val gly val arg ile tyr val asp ala val ile asn his
UGC AAC AAU GUU GGU GUC CGG AUU UAU GUG GAU GCU GUC AUU AAU CAC

met cys gly ser gly asn ser ala gly thr his ser thr cys gly ser
AUG UGU GGC UCG GGC AAU AGU GCA GGA ACA CAC AGU ACC UGU GGA AGU

tyr phe asn pro asn asn arg glu phe ser ala val pro tyr ser ala
UAC UUC AAU CCU AAU AAC AGG GAA UUC UCA GCA GAA UUC CCA UAC UCU GCU

trp tyr phe asn asp asn lys cys asn gly glu ile asn asn tyr asn
UGG UAU UUU AAC GAU AAU AAA UGU AAU GGA GAA AUU AAU AAC UAC AAU

asp ala asn gln val arg asn cys arg leu ser gly leu leu asp leu
GAU GCC AAU CAG GUC AGA AAU UGU CGU CUG UCU GGC CUU CUG GAU CUU

ala leu asp lys asp tyr val arg thr lys val ala asp tyr met asn
GCA CUC GAU AAA GAU UAU GUU CGA ACC AAG GUG GCU GAC UAU AUG AAC

asn leu ile asp ile gly val ala gly phe arg leu asp ala ala lys
AAU CUC AUU GAC AUU GGU GUA GCA GGG UUC AGA CUU GAU GCU GCU AAG

his met trp pro gly asp ile lys ala val leu asp lys leu his asn
CAC AUG UGG CCU GGA GAC AUA AAG GCA GUU UUG GAC AAA CUA CAU AAU

leu asn thr lys trp phe ser gln gly ser arg pro phe ile phe gln
CUA AAU ACA AAA UGG UUC UCC CAA GGA AGC AGA CCU UUC AUU UUC CAA

glu val ile asp leu gly gly glu ala ile lys gly ser glu tyr phe
GAG GUC AUU GAU CUU GGU GGU GAA GCA AUU AAA GGU AGU GAG UAC UUU

gly asn gly arg val thr glu phe lys tyr gly ala lys leu gly thr
GGA AAU GGC CGC GUG ACA GAA UUC AAG UAU GGU GCA AAA CUU GGC ACA

val ile arg lys trp asn gly glu lys met ser tyr leu lys asn trp
GUU AUU CGC AAA UGG AAU GGA GAG AAG AUG UCU UAC UUA AAG AAC UGG

gly glu gly trp gly phe val pro thr asp arg ala leu val phe val
GGA GAA GGU UGG GGU UUU GUG CCU ACU GAC AGA GCC CUU GUG UUU GUG

asp asn his asp asn gln arg gly his gly ala gly gly ala ser ile
GAC AAC CAU GAC AAU CAG CGA GGA CAU GGU GCU GGA GGA GCA UCC AUC

leu thr phe trp asp ala arg met tyr lys met ala val gly phe met
CUG ACA UUC UGG GAU GCU AGA AUG UAU AAA AUG GCA GUU GGA UUU AUG

leu ala his pro tyr gly phe thr arg val met ser ser tyr arg arg
UUG GCU CAU CCU UAU GGA UUC ACC AGA GUA AUG UCA AGU UAC CGA AGG

thr arg asn phe gln asn gly lys asp val asn asp trp ile gly pro
ACA AGA AAU UUC CAG AAU GGA AAA GAU GUG AAU GAC UGG AUU GGA CCA

pro asn asn asn gly val thr lys glu val thr ile asn pro asp thr
CCU AAU AAC AAU GGA GUA ACA AAA GAA GUG ACC AUU AAU CCA GAC ACU

thr cys gly asn asp trp val cys glu his arg trp arg gln ile arg
ACU UGU GGC AAU GAC UGG GUC UGU GAA CAU CGA UGG CGU CAA AUC AGG

asn met val ala phe arg asn val val asn gly gln pro phe ala asn
AAC AUG GUU GCC UUC AGG AAU GUA GUC AAC GGU CAG CCU UUU GCA AAC

trp trp asp asn gly ser asn gln val ala phe ser arg gly asn arg
UGG UGG GAU AAU GGC AGC AAC CAA GUG GCU UUU AGC AGA GGA AAC AGA

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440      450      460      470      480      490      500      503
gly phe ile val phe asn asn asp asp trp ala leu ser ser thr leu
GGA UUC AUU GUC UUU AAC AAU GAU GAC UGG GCU UUG UCA AGC ACU CUA

gln thr gly leu pro ala gly thr tyr cys asp val ile ser gly asp
CAG ACU GGU CUU CCU GCU GGC ACA UAC UGU GAU GUC AUU UCA GGA GAU

lys val asn gly asn cys thr gly leu lys val asn val gly ser asp
AAA GUC AAU GGC AAU UGC ACU GGA CUU AAA GUA AAU GUU GGC AGU GAU

gly lys ala his phe ser ile ser asn ser ala glu asp pro phe ile
GGC AAA GCU CAC CAC UUC UCU AUU AGU AAC UCU GCU GAA GAC CCA UUC AUU

ala ile his ala asp ser lys leu OC
GCA AUC CAU GCC GAC UCA AAG UUG UAA GAGUCAAAUUAAGAGAUUAGAUAUCA
GCACCA

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Fig. 2 The nucleotide sequence of rat pancreatic amylase mRNA. The sequence was determined from the double-stranded cDNA inserts of pcXP38 and pcXP100 (Fig. 1) by the method of Maxam and Gilbert¹⁸. The predicted amino acid sequence is indicated. The divergent heptanucleotide sequence AAUUAUA is underlined. The single nucleotide discrepancy between the double strands of pcXP38 and pcXP100 is circled.

general feature of mRNAs encoding the pancreatic secretory proteins because the 3'-untranslated region of proelastase mRNA is 289 nucleotides long¹⁷. The short 3'-untranslated region requires that functionally important sequences near the 3' end of amylase mRNA be either less than 35 nucleotides or contained in the coding region. The heptanucleotide, AAUUAUA is present in this region centred 23 nucleotides from the poly(A). This sequence appears to be the first detected variant of the hexanucleotide sequence, AAUAAA, found approximately 20 nucleotides from the poly(A) in every polyadenylated RNA sequenced to date. The variation of this sequence probably reflects a flexibility in the sequence requirement at this site rather than a modulation of some aspect of the metabolism of exocrine mRNAs since the hexanucleotide, AAUAAA, is conserved in proelastase mRNA¹⁷. Interestingly, the heptanucleotide AAUUAUA has also recently been found in anglerfish pancreatic somatostatin mRNA³².

Structural domains in amylase

Several experimental observations suggest that the amylase molecule is arranged in two similar domains. Mammalian amylase can bind two molecules of the substrates glycogen³³ and maltotriose³⁴ and at least two molecules of the substrate analogue β -cyclodextran³⁵. An endogenous protease^{36,37} cleaves amylase into two polypeptides of approximately equal size

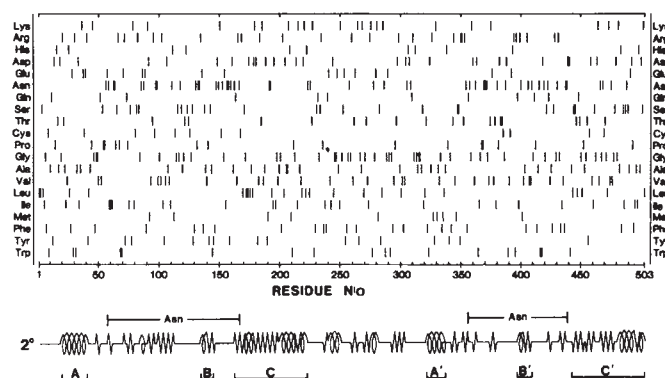


Fig. 3 The amino acid distribution and predicted secondary structure of amylase. Vertical bars indicate the sequence position of each amino-acid residue. Regions of α -helix (coiled lines) and β -sheet (zigzag lines) were predicted using the methods of Garnier *et al.*⁴² and Chou and Fasman⁴³. The former method also predicts β -turns and random coil; however for clarity these have not been included.

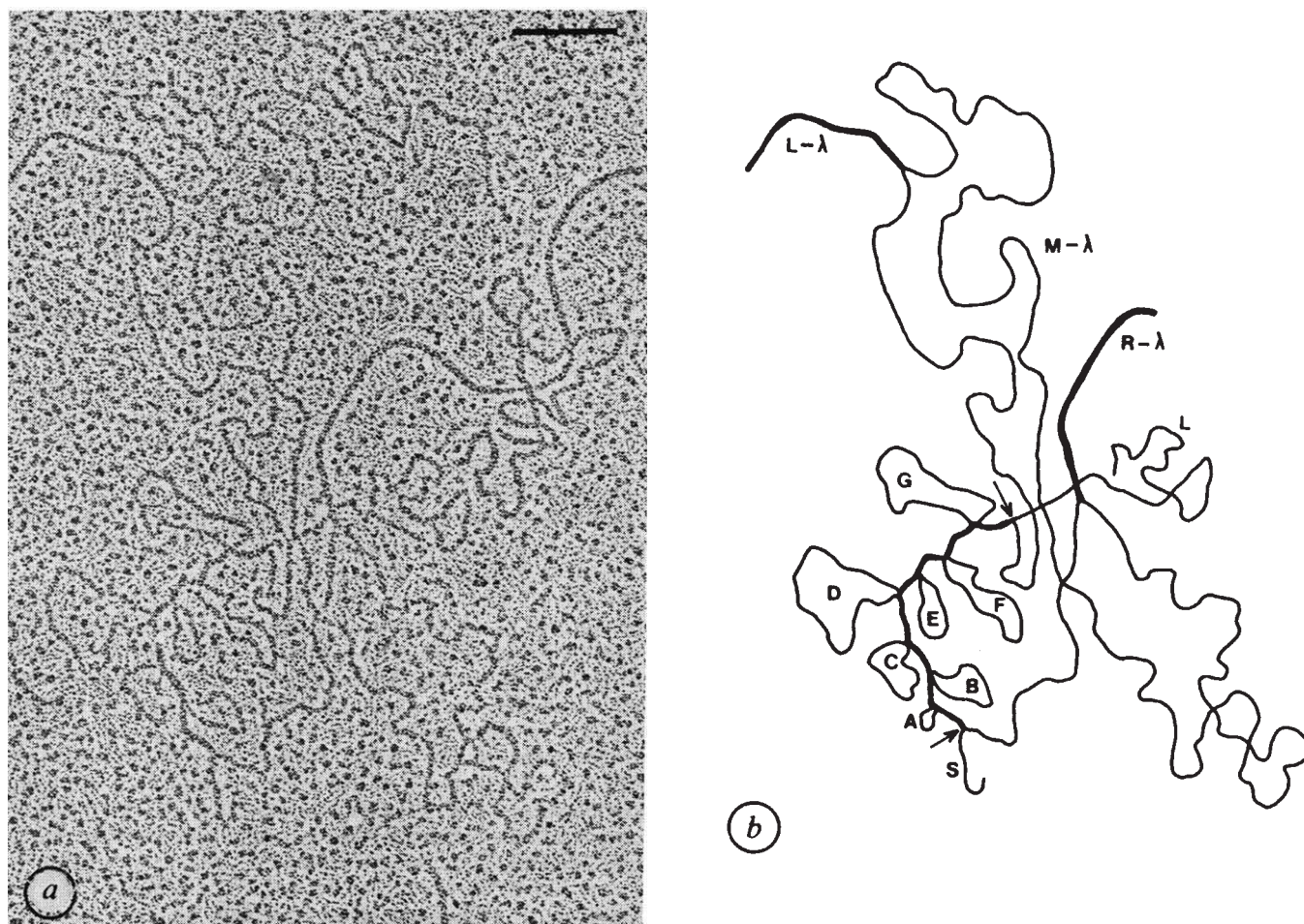


Fig. 4 Heteroduplex analysis of the sequence organization of a rat amylase gene. *a*, An electron micrograph of a heteroduplex structure formed by annealing recombinant λ DNA containing the rat genomic DNA fragment 237 with the amylase cDNA plasmid pcXP101 and with wild-type Charon 4A DNA. pcXP101 was constructed by ligating together the amylase cDNA inserts in pcXP100 and pcXP38 at the unique *Ava*II restriction endonuclease site within the region of overlap (see Fig. 1). pcXP38 had originally been cloned in the *Hind*III site of pBR322 using *Hind*III restriction site oligonucleotide linkers, whereas pcXP100 had been cloned in the *Pst*I site of pBR322 by the G + C tailing method¹⁷. To clone the combined cDNA fragments into pBR322 the pcXP100 cDNA insert was digested with *S*₁ nuclease to produce blunt ends and then ligated with *Hind*III linkers⁵⁴. The DNA was then digested with *Ava*II and ligated to the *Ava*II-digested pcXP38 cDNA insert. The correct ligation product was size-selected by agarose gel electrophoresis and after digestion with *Hind*III endonuclease, recloned in the *Hind*III site of pBR322. Successful construction of the desired cDNA was confirmed by restriction endonuclease analysis. For heteroduplex analysis the three DNAs with a 5–10 fold molar excess of cDNA were mixed and denatured in 0.1 M NaOH, the pH of the DNA mixture was then lowered to 8.4 with Tris to a final molarity of 0.1 M and made 50% in formamide. The DNAs were then incubated at 25 °C for 6 h to obtain about 50% of the molecules as heteroduplexes. The DNA was then prepared for electron microscopy as described by Fergusson and Davis⁵⁵. The cDNA plasmid pcXP101 as well as the Charon 4A DNA arms flanking the genomic DNA insert were used as size markers. Scale bar, 0.2 μ m. *b*, An interpretive drawing of the heteroduplex structure in *a*. Intervening sequences are designated A–G in the direction of transcription of the gene. Arrows delimit the region of homology between the genomic fragment and the cDNA. Single-stranded DNA tails present at the ends of the gene sequences marked S and L represent the short and long tails of pBR322, respectively. The double-stranded DNAs situated at the ends of the heteroduplex structures marked L- λ and R- λ represent the left and right arms of Charon 4A DNA, respectively, and M- λ represents the middle region of the wild-type Charon 4A DNA.

which exhibit undiminished enzyme activity and nearly identical tryptic peptide maps. Hydrodynamic and low angle X-ray scattering measurements³⁸ as well as preliminary X-ray crystallographic data^{39,40} indicate a molecule consisting of two similar but non-identical lobes separated by a cleft. Although in need of confirmation by X-ray crystallography, significant structural homology between the halves of the proposed amino acid sequence (divided between residues 270 and 271) is suggested by comparison of the halves of the sequence according to the method of McLachlan⁴¹ and by the secondary structure predicted from the proposed sequence according to the methods of Garnier *et al.*⁴² and Chou and Fasman (ref. 43 and Fig. 3). Predicted regions of α -helix and β -sheet designated A, B and C (Fig. 3) in the amino-terminal half of the sequence are repeated as similar but non-identical regions A', B' and C' in the carboxyl-terminal half. Asparagine-rich clusters are present in each half and are located symmetrically between regions A and C and A'

and C'. A Pro-Pro sequence is present in each half of the sequence (positions 54–55 and 367–368) located similarly with respect to regions A and A'. A glycine-rich region (21% glycine residues extending from position 245 to 316) separates the two putative domains. The sequence Arg-Lys is present at positions 274 and 275 in the approximate middle of the molecule, and may correspond to the protease-hypersensitive site observed by Robyt *et al.*³⁶.

A comparison of the two putative domains reveals no significant sequence homology between these regions at either the amino acid or nucleotide level. Conservation of intramolecular structural homology despite retention of little or no amino acid sequence homology has been observed in penicillopepsin⁴⁴ and rhodanese⁴⁵. In view of the lack of nucleotide sequence homology between the putative domains the origin of the two domain structure, which could be via fusion of tandemly duplicated primordial genes⁴⁶, remains an open question.

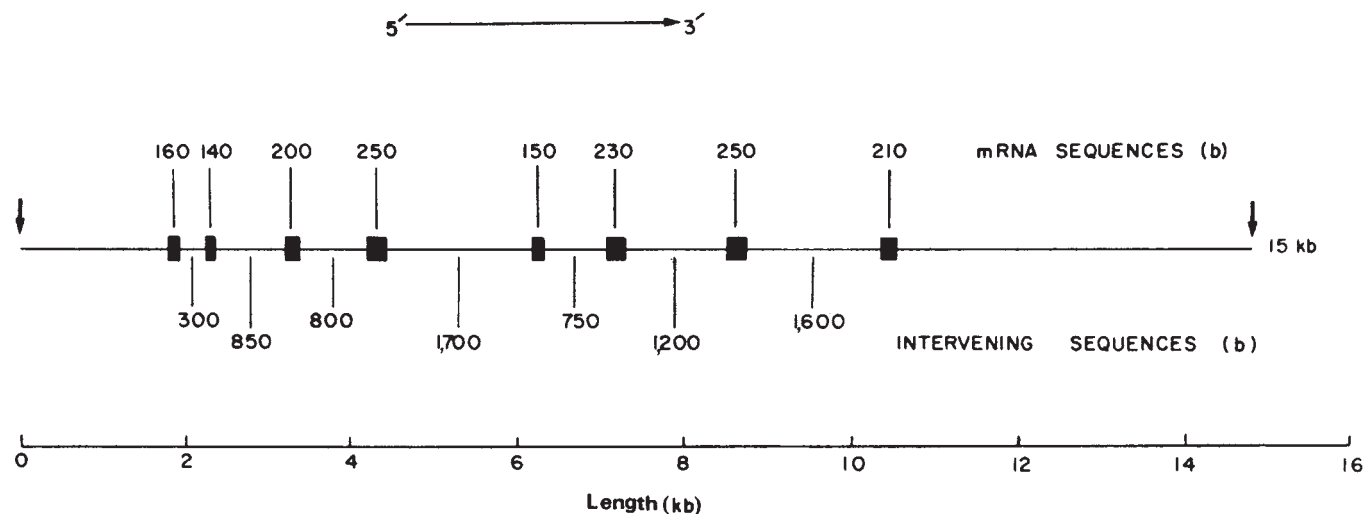


Fig. 5 A schematic representation of the organization of amylase gene sequences in the genomic DNA fragment 237. The lengths of the mRNA (cDNA) and intervening sequences are mean values derived from measurements of at least four heteroduplex structures. Standard deviations range from $\pm 5\%$ for the larger to $\pm 20\%$ for the smallest measurements. λ Charon 4A DNA sequences are not shown here and the arrows indicate the ends of the genomic fragment. kb, kilobases.

Amylase gene structure

Using the cDNA inserts of pcXP100 and pcXP38 as probes, we have screened a rat genomic DNA- λ Charon 4A DNA library (provided by T. Sargent, R. B. Wallace and J. Bonner⁴⁷) for phage containing rat amylase gene sequences by the method of Benton and Davis⁴⁸. Thirty-nine positive recombinant phage were obtained from 2×10^6 plaques and were subsequently purified and analysed.

The organization of amylase gene sequences within these fragments was determined by restriction endonuclease analysis and electron microscopic examination of heteroduplexes formed by annealing the DNA of the recombinant phage with the amylase cDNA plasmid designated pcXP101. This plasmid was constructed by joining the cDNA inserts in pcXP100 and pcXP38 at a common, unique restriction endonuclease site as detailed in the legend to Fig. 4. The combined cDNA was recloned in pBR322 and its 5' to 3' orientation within the plasmid was determined by restriction endonuclease analysis. On digestion of pcXP101 with *SalI*, a linear molecule with a short strand of pBR322 sequences (0.6 kilobase pairs) at the 5' end and a long strand (3.7 kilobase pairs) at the 3' end of the cDNA insert is formed. Amylase genomic sequences could then be orientated after hybridization to this probe by measuring the pBR322 tails present at the ends of the genomic DNA-cDNA hybrid. Wild-type Charon 4A DNA was also included in the heteroduplex mixture to delimit the left and right λ arms which flank the genomic DNA fragments. Genomic sequences flanking the amylase gene sequences could then be identified.

The 39 positive fragments obtained from the library appear to represent nine distinct amylase genes. However, the overall sequence organization as determined by heteroduplex analysis suggests that these genes are all related to a common amylase gene motif. Figure 4a and b show, respectively, an electron micrograph and the corresponding interpretative drawing of a heteroduplex structure of a gene which exhibits the structural features found in the other gene fragments. This genomic fragment contains cDNA sequences that are interrupted by seven detectable intervening sequences; 5' and 3' flanking regions are also present. Estimates of the lengths of these regions are given in Fig. 5. Amylase cDNA sequences are present within a region of 9 kilobases and range in length from approximately 140 to 250 bases. The correspondence of the aggregate lengths of these sequences ($1,600 \pm 100$ bases) with the length of the cDNA probe (1,547 bases), suggests that the entire length of the cDNA is hybridized to the genomic fragment in the heteroduplex. The lengths of the intervening sequences range from approximately 300 to 1,700 bases. Flanking regions extend 2 and 4 kilobases

from the 5' and 3' ends, respectively, of the amylase gene sequence. We emphasize that the present experiments do not detect sequences at the extreme 5' end of the mRNA since these sequences are missing in the cDNA probe; intervening sequences have been observed in this region in a number of eukaryotic genes^{20,49-53}. Since the 5' flanking region is 2 kilobases long it is likely that sequences corresponding to the 5' end of the mRNA are present in this genomic fragment. More extensive 5' flanking regions, up to 12 kilobases long, are present in other genomic fragments. A sequence analysis of this region should prove useful in analysing tissue-specific gene expression.

Since the library used for this analysis was constructed from a single rat⁴⁷, the nine cloned amylase sequences must represent at least five non-allelic genes or pseudogenes. In fact, the nine sequences can be arranged into four closely related pairs by the patterns of restriction endonuclease digestion. With this isolation and characterization of the cDNA and genes it is now possible to envisage a more penetrating analysis of their selective expression in various differentiated cells.

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LETTERS

Origin of the 5 March 1979 γ -ray transient: a vibrating neutron star

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An unusual γ -ray transient was observed on 5 March 1979, with 12 different instruments on 9 different spacecraft^{1–4}. Most of these instruments are incorporated into an interplanetary γ -ray burst network. The source position of the 5 March transient, determined by this network to an accuracy⁴ of $\sim 1 \times 2$ arc min, is consistent with the direction of the supernova remnant N49 in the Large Magellanic Cloud (LMC). Subsequent analysis⁵ of the data, by narrowing the source error box to an area of about 6 by 30 arc s inside the supernova remnant, considerably strengthens this identification. We propose that a vibrating neutron star in the LMC is the source of the 5 March transient. This may be both the first detection of a vibrating neutron star and indirect evidence for gravitation radiation.

In addition to its identification, the 5 March transient has several unique characteristics that set it apart^{3,6} from the general class of γ -ray bursts: the peak flux of $\sim 10^{-3}$ erg cm⁻² s⁻¹ in the impulsive phase of the event is an order of magnitude greater than that of any other γ -ray burst. At the distance of the LMC (55 kpc), this implies a luminosity of $\sim 3 \times 10^{44}$ erg s⁻¹. The duration of the impulsive phase, ~ 0.15 s, is also shorter than that of most γ -ray bursts which last for several seconds. The 5 March impulsive energy release, $\sim 5 \times 10^{43}$ erg, would thus be larger by more than five orders of magnitude than typical γ -ray burst energies, whose probable galactic origin⁷ requires energy releases of only $\sim 10^{38}$ erg.

The exceedingly short rise time of this impulsive phase, $< 2 \times 10^{-4}$ s, suggests that the size of the emission region does not exceed ~ 60 km, the light travel distance for this time. That

the source may in fact be a neutron star is implied by the energy spectrum¹ of the impulsive phase (Fig. 1) which shows a broad feature centred at ~ 0.4 – 0.43 MeV probably due to the gravitational redshift of 0.511 MeV electron-positron annihilation radiation. This radiation should be produced close to the surface of a neutron star with $z = (1 - 2GM/Rc^2)^{-1/2} - 1 \approx 0.19$ to 0.28 where M and R are the mass and radius of the star. Similar redshifts have been reported for at least one other γ -ray burst^{8,9} and longer duration γ -ray transients^{10,11}.

A neutron star origin for the 5 March event is also suggested by the observed^{1–3} pulsations which follow the impulsive phase for at least 3 min. The pulse period is 8.0 ± 0.05 s, and the average pulsed flux, $\sim 10^{-5}$ erg cm⁻² s⁻¹, decreases exponentially with a characteristic time of ~ 50 s. These pulsations could be due to rotation, although the period seems to be too long for a young ($\leq 10^4$ yr) neutron star in N49. Such pulsations have not been seen from any other γ -ray burst. The 5 March transient was also followed by three other outbursts⁹, apparently from the same source direction, on 6 March, 4 April and 24 April. The peak intensities of these outbursts, however, were much lower than that of the 5 March event, and decreased with each burst.

The energy spectrum of the 5 March event is much softer than that of typical¹² γ -ray bursts. In fact, the bulk of the emission of the 5 March event is in the lower energy (≤ 0.2 MeV) part of the spectrum which is significantly steeper than that of other γ -ray bursts^{13,14} in this energy range.

Although the burst source position strongly suggests association with N49, there are several problems if the source were a neutron star in the LMC. These concern the origin and rapid release of the observed energy, and the very high efficiency of the radiation mechanism. The required mechanism apparently violates the black-body limit in that it must provide a very large luminosity at relatively low photon energies from a small emitting area. Indeed, several authors, using arguments based on the Eddington limit for accretion models¹, on the opacity of γ rays to Compton scattering and pair production^{15,16}, and on the slow diffusion of photons to the neutron star surface¹⁷, have concluded that the source of the 5 March transient should be closer than a few hundred parsec.

Here we consider that the source of the 5 March transient is in the LMC, based on the rather convincing positional identification with N49. The arguments against an LMC origin based on radiation mechanisms are removed by the e^+e^- synchrotron cooling and annihilation model presented elsewhere¹⁸ and briefly discussed here. Regarding the energy source, its very large magnitude requires that it be interior to the star, most likely of gravitational origin. The transport of this energy cannot be by photon diffusion. We propose instead that

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neutron star vibrations could, in a coherent and rapid fashion, communicate the internal energy to the upper crust and atmosphere. Decay of these vibrations by gravitational radiation¹⁹ would then account for the duration of the impulsive phase, ~ 0.15 s. An LMC origin for the 5 March transient is not in conflict^{6,20} with a galactic origin for the more common γ -ray bursts, as these probably belong to a different class of transients than does the 5 March event.

The basic source of energy in the 5 March transient is not clear, but it could be gravitational energy released by a phase transition²¹ in the interior. In this case, the energy should be communicated to the surface in a time comparable to the sound-wave crossing time through the neutron star. As the speed of sound in neutron star matter is²² of the order c , this time is less than the upper limit in the rise time of the burst. The released gravitational energy could be much larger than observed in γ rays, but the bulk of this energy is not expected to be emitted as electromagnetic radiation. A large fraction of the released energy could in fact be in vibrations²², $E_{\text{vib}} \cong 10^{53}$ erg $(\delta R/R)^2$, where δR is the vibrational amplitude. For example, if $\delta R/R \sim 10^{-3}$, $E_{\text{vib}} \sim 10^{47}$ erg, in which case conversion of only a small fraction (10^{-3}) of this energy into γ rays would be sufficient to account for the observations.

The strong magnetic fields that are tied to the neutron star play an important part in this conversion. As these fields oscillate together with the surface, they should produce wave motions which would accelerate particles in the atmosphere. In particular, the bulk motions may be converted into magneto-acoustic waves, the high frequency portions of which dissipate in the atmosphere and heat it. Because of the relatively small number of ambient atmospheric particles, this heating would rapidly raise the temperature to a value ($\leq 10^9$ K) at which e^+e^- pair production sets in. Further heating increases the radiation density, and the atmosphere becomes radiation dominated. In this case, the density of pairs which coexist with the radiation greatly exceeds that of the ambient matter. As this hot pair plasma attempts to escape from the neutron star, it pushes the magnetic field outwards. But the field, being tied to the star, is

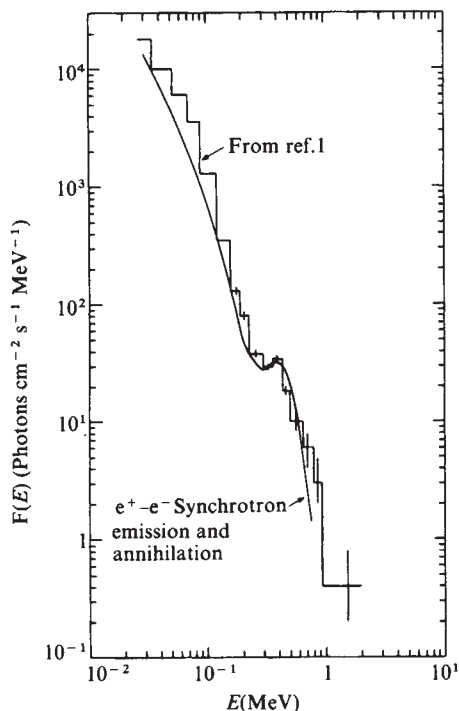


Fig. 1 Energy spectrum of the impulsive phase of the 5 March transient measured¹ during the first 4 s of the event. The photon flux of the impulsive phase is larger than that shown in the figure by a factor of 4/0.15, where 0.15 s is the duration of the impulsive phase. The curve is the calculated¹⁸ spectrum in the e^+e^- synchrotron cooling and annihilation model.

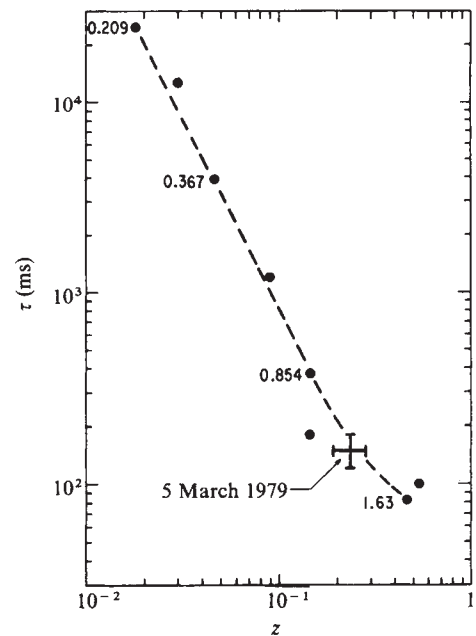


Fig. 2 The calculated²⁶ (●) quadrupole gravitational radiation damping time versus gravitational redshift for neutron stars. The dashed curve connects cases having the same equation of state and the numerical values are neutron star masses in units of $M_\odot$. The datum point is from γ -ray observations of the 5 March transient¹⁻³.

compressed, and in the resulting strong field regions the pairs are rapidly cooled by synchrotron radiation.

The e^+e^- synchrotron cooling and annihilation model¹⁸, which we now briefly describe, can account (see Fig. 1) for the observed impulsive spectrum. The essential feature of this model is the radiation dominated e^+e^- pair plasma. Even though its annihilation time is very short ($\sim 10^{-12}$ s for the parameters¹⁸ of the 5 March transient), this plasma can maintain itself because the annihilation photons have enough energy to produce new pairs. The energy spectrum of the coexisting radiation, however, is much harder than the observed spectrum. But in regions of strong magnetic field, the pairs can lose their kinetic energies before they annihilate and this converts the hot ($\sim$ MeV) photon-pair plasma into cooler radiation ($\sim$ tens of keV) that is devoid of pairs. This conversion takes place in a layer whose thickness does not exceed the photon-photon pair production mean free path. This outer skin layer is also the effective radiating volume of the star. The magnetic field, the pair density and the thickness of the layer are $\geq 10^{11}$ G, $\sim 2 \times 10^{26}$ cm⁻³, and 0.1 mm, respectively¹⁸. The curve in Fig. 1 is the calculated¹⁸ spectrum produced in the layer: the low energy part (≥ 0.2 MeV) is the synchrotron radiation of the pairs, while the high energy part is their annihilation radiation.

The stability and confinement of the radiating layer is an essential requirement of the model, because its absence would lead to relativistic outward expansion that would result in blueshifted radiation rather than the redshifted emission needed to account for the observations. We believe that the magnetic field does play a major role in the confinement, as the energy density in the field is at least as large as that of the e^+e^- plasma. In particular, confinement may be achieved in the highly compressed magnetic field ahead of the pair plasma. The positive (outward) gradient of the field should exert an inward confining force on the plasma. Possible instabilities, such as the exchange instability, and a radiation-pressure driven Rayleigh-Taylor instability, may be stabilized by the continuous transformation of particles into photons and vice versa, as well as by the strongly sheared field.

The conversion of the neutron star vibrational energy into γ rays should go on as long as the star continues to vibrate. The principal damping mechanism of the vibrations should be

gravitational radiation¹⁹. Neutrino emission²³ is an important damping mechanism only for very young hot neutron stars, while viscous effects (the Urca process and a process²⁴ involving Σ hyperons) are slower²² than gravitational radiation damping. Escaping shock waves can also damp the vibrations²², but the loss rate due to shocks should not exceed the observed electromagnetic emission which in our model constitutes only a small fraction of the vibrational energy.

Gravitational radiation is expected to damp the quadrupole and higher mode vibrations on time scales ranging from about 0.1 to 10 s, depending on the properties of the neutron star^{25,26}. Using the calculations of Detweiler²⁶, we plot in Fig. 2 the damping time τ as a function of redshift z , where the dashed curve connects points obtained from the same equation of state. The numerical values next to these points are the corresponding neutron star masses in units of $M_{\odot}$. The 5 March 1979 datum point shows the observed¹⁻³ duration of the impulsive phase (120–180 ms) versus the approximate redshift¹. The implied neutron star mass is ~ 1 – $1.3 M_{\odot}$, its radius ~ 10 km, and its quadrupole vibrational frequency ~ 0.4 ms. The sensitivities of the present gravitational wave detectors were insufficient to observe the expected gravitational radiation (J. A. Tyson, personal communication), and the time resolution of the γ -ray instruments were insufficient to resolve the expected vibrational period in the impulsive emission.

Although gravitational radiation is very effective in damping the quadrupole and higher mode vibrations, it will damp the radial oscillations more slowly^{19,22}. The Σ^- process²⁴, however, could damp the radial vibrations on a time scale of ~ 50 s, the characteristic decay time of the pulsed phase of the 5 March transient. Thus, the energy source for this phase as well could be neutron star vibrations. The observations would, therefore, imply that the γ -ray luminosity produced by the radial oscillations is smaller than that of the higher modes. This could result from asymmetric excitation of the vibrations which would put more energy into nonradial vibrations. Also, due to its small scale height, the atmosphere is expected to couple better with the higher modes than with radial oscillations. The 8-s period of the pulsed emission, however, is not likely to be a vibrational period. It may be caused by the rotation of the neutron star, although neutron star precession is an alternative explanation (K. Brecher, personal communication). The subsequent weaker bursts, which seem to come from the same source on 6 March, 4 and 24 April, may be aftershocks of the 5 March burst if it indeed results from a major quake in the neutron star interior.

There are, of course, several unresolved aspects of the model that clearly need further study. These include the more precise description of the source of energy of the burst, the nature and efficiency of vibrational heating, the stability of the thin emission region, and the origin of the 8-s pulsed emission. The present general outline of a model for the 5 March transient can account for both the enormous luminosity and the complex temporal and spectral features of the burst by emission from a neutron star in the LMC.

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Low energy γ rays from PSR1822–09

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Pulsed γ -ray emission in the MeV range (80 keV–6 MeV) has been clearly established^{1–4} for the Crab pulsar PSR0531+21 ($P=33$ ms). The energy spectrum of the pulsed component follows a power law from the X-ray (100 keV) to the high energy γ -ray range (2 GeV) with an index close to 2.1 (ref. 5). Positive detection of pulsed γ ray emission from the Vela pulsar PSR0833–45 ($P=89$ ms) has been claimed in the 10–30 MeV region by Albats *et al.*⁶, and clearly established⁵ in the high energy range 50 MeV–3 GeV with a power law index of 1.9. This pulsar was also observed in the X-ray range but with apparently conflicting results (ref. 7 and refs therein), some authors reporting positive detection while other have upper limits below the reported values. Other radio pulsars, especially PSR1822–09, and PSR1747–46 have been investigated at energies above ~ 50 MeV by the satellites SAS 2^{8,9} and COS B¹⁰. We report here observations of PSR1822–09 ($P=769$ ms) between 80 keV and 6 MeV at balloon altitudes.

The equipment used, which is described in detail elsewhere¹¹, consisted of a 10-cm diameter CsI(Tl) crystal (78.5-cm² area), 2.5-cm thick, shielded against background by an active NaI(Tl) well 5-cm thick and 8-cm high blocked off at the bottom by 2 NaI(Tl) disks, a total of 10-cm thick whose diameters were the same as the outside diameter of the well (20 cm). The detector field of view, defined by the anticoincidence well, is a cone of half angle 45°. A plastic scintillator 0.5-cm thick was placed against the CsI(Tl) detector to block out charged particles entering the aperture. In the absence of an anticoincidence signal, the CsI(Tl) pulses were analysed by two 128 channel ADCs¹² covering the energy ranges of 80–1,126 keV and 1.5–6 MeV. All events detected during the flight were dated with a ground based clock to within 0.1 ms. The stability of the datation process was checked using a simultaneous recording of a 10⁵ Hz oscillator. Gain variations, checked in flight by the observation of the atmospheric 511-keV line, are $<1\%$.

The response of the detector in both energy and angle with respect to the viewing axis was determined in the laboratory using 14 calibrated radioactive sources whose energies covered the range 80 keV–8 MeV. This calibration gives a response matrix for the detector which is used to find the incident photon spectrum using the detected electron spectrum¹³.

The telescope, mounted vertically, was launched from Guaratinguetá, Brazil (latitude 22°48' S, rigidity 11.7 GV) on 12 February 1977. During this flight, which lasted 5 h at a ceiling altitude of 2.4 g cm^{–2}, the galactic disk was observed¹⁴. As the pulsar PSR1822–09 was also in the field of view, the data were analysed to see whether the pulsar signature could be detected.

We have divided the data into three energy intervals. The pulsar light curve in each energy range, was constructed by folding the data, consisting of all the events, modulo the

apparent period, into 10 phase bins, each event being placed in one of the bins as a function of its arrival time. The apparent period was found from the radio values of P and $\dot{P}$ given by Taylor and Manchester¹⁵ after extrapolation to the date of the flight (JD = 2443187.0), and correction for the Doppler effect due to the movement of the Earth; the source was taken to be at the right ascension given by Manchester *et al.*¹⁶ and the declination given by Taylor and Manchester¹⁵. Only the data taken between zenith angles 13° and 33° were used, which correspond to times between 10:45 and 14:00 UT; the effective exposure at 1 MeV was $3.9 \times 10^5 \text{ cm}^2 \text{ s}^{-1}$.

Figure 1 shows the light curve obtained between 454 and 1,126 keV. The χ^2 value for this distribution is 16 for 9 d.f., corresponding to a probability $P(\chi^2 \geq 16)$ of 0.07. A Monte Carlo simulation was used to evaluate the overall probability resulting from the three phase histograms obtained using the energy ranges: 80–454 keV ($P=0.4$); 454–1,126 keV ($P=0.07$); and 1.5–6 MeV ($P=0.15$). An overall probability of <1% was obtained. The light curve in the energy range 454–1,126 keV has two characteristic peaks separated by a phase of 0.5 ± 0.1 . This structure is also found for PSR0531+21 and for PSR0833-45, above a γ -ray energy of 50 MeV. From this comparison, the two peaks labelled A and B in Fig. 1 have been chosen as pulsed bins in the light curve. The background level is defined using the six remaining bins. With respect to this level, the number of pulsed events (in A and B) is at a 3.4 standard deviation above this background level. Peak A is $\sim 230 \text{ ms}$ wide; peak B is 77 ms wide. The intensity ratio between peaks A and B is ~ 3 .

The energy spectrum of the pulsed events was constructed by distributing equally all the data (80 keV–6 MeV) into four energy channels. The incident photon spectrum, corrected for atmospheric absorption, is shown in Fig. 2. A positive detection is observed only for the two channels: 300–530 keV ($(3.1 \pm 1.2) \times 10^{-3} \text{ photons cm}^{-2} \text{ s}^{-1} \text{ MeV}^{-1}$) and 530–1,126 keV ($(3.8 \pm 1.2) \times 10^{-3} \text{ photons cm}^{-2} \text{ s}^{-1} \text{ MeV}^{-1}$). The pulsar was not detected either between 80 and 300 keV (3σ upper limit 4.4×10^{-3}

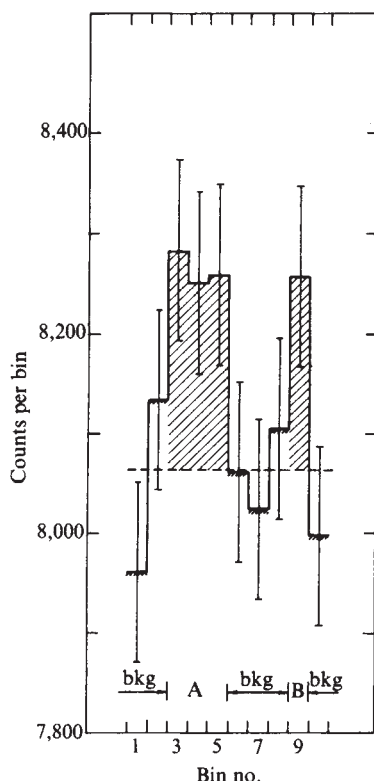


Fig. 1 Light curve observed for PSR1822-09 in the energy range 454–1,126 keV. $P(\chi^2 \geq 16/9 \text{ d.f.}) = 0.07$. The dashed line represents the background level.

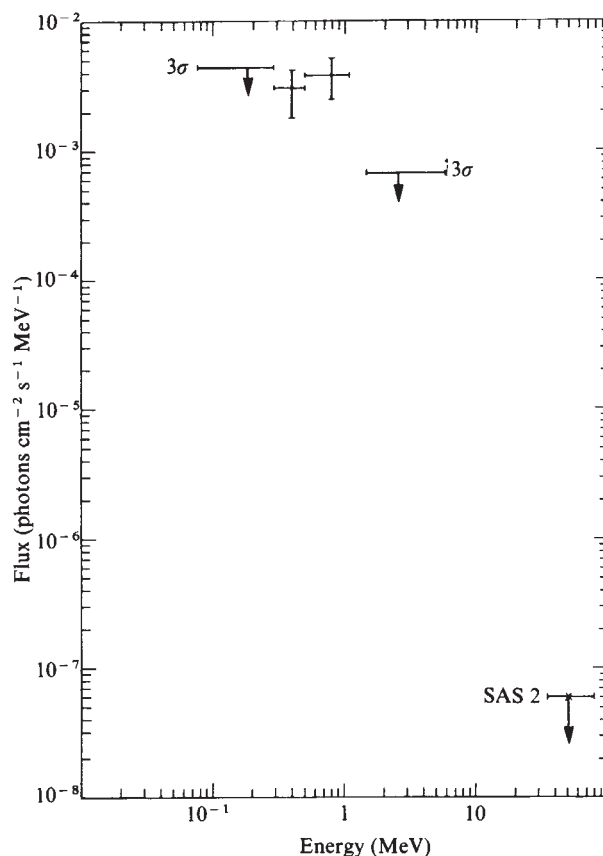


Fig. 2 Energy spectrum for PSR1822-09 using the pulsed events in the two peaks (see text).

photons $\text{cm}^{-2} \text{ s}^{-1} \text{ MeV}^{-1}$) or between 1.5 and 6 MeV (3σ upper limit $6.7 \times 10^{-4} \text{ photons cm}^{-2} \text{ s}^{-1} \text{ MeV}^{-1}$). Figure 2 also shows 3σ upper limits for these two channels, as well as the upper limit between 30 and 80 MeV taking the upper limit of the flux above 35 MeV ($2.7 \times 10^{-6} \text{ photons cm}^{-2} \text{ s}^{-1}$), from SAS 2⁸, and assuming an $E^{-2.4}$ spectrum.

This spectrum is very different from that observed in the case of the Crab pulsar which is a power law equal to -2.1 . It shows a remarkable break around 1 MeV, the index being smaller than -2 above this energy taking into account the SAS 2 upper limit, and close to zero below 1 MeV, if we consider the upper limit between 80 and 300 keV.

Various theories have been invoked to explain pulsar emission. Some of them consider that this emission is localized near the crust of the neutron star^{17,18} and others, at the light cylinder^{19,20}. In the first model, the high energy particles are accelerated near the crust of the neutron star and propagate along the open magnetic field lines. These particles produce high energy photons by curvature radiation. The primary γ photons can be absorbed by pair production when they cross the magnetic field lines. The secondary particles created by this process emit instantaneously a flux of γ -ray photons by the synchrotron process. The resulting spectral shape depends on the pulsar period, the Lorentz factor of the primary electrons (positrons) and the intensity of the magnetic field at the neutron star pole. Above 1 MeV, the observed spectral index is then consistent with a Lorentz factor for the primary particles of $\sim 10^7$ or less.

Around 1 MeV our data show a clear break in the spectrum: this could be due to a synchrotron self-absorption process or to a pair-production absorption process. Our results imply that the most important part of the energy emitted by PSR1822-09 is emitted in the MeV region. Other observations in this energy range are necessary to confirm this result and to understand better the geometry of the γ emission for this pulsar.

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Discussion of the Pioneer 11 observations of the F ring of Saturn

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The photopolarimetric and charged particle data from the Pioneer 11 Saturn encounter were used as diagnostic tools in a preliminary analysis of the physical nature of the F ring of Saturn. It is argued that the F ring may have two components, with the outer one composed of solid particles of centimetre-diameter and the inner one of micrometre-sized dust particles. Also, the outer component as scanned by the charged particle detectors may have two rings instead of one. The double-ring option, if verified, could have interesting implications for the dynamical origin of the planetary ring systems.

Among the many exciting new results from the recent Pioneer 11 flyby of Saturn^{1,2} one of the major findings is the discovery of a new (F) ring located at $2.33 R_s$ (Saturn radius) just outside the A ring by the Imaging Photopolarimeter (IPP) team³. The absorption effects of trapped charged particles by this ring were subsequently detected by the particle experiments⁴⁻⁷. According to the IPP observations, the F ring has a width of no more than 800 km and an optical depth of $\leq 2 \times 10^{-3}$. In comparison with the whole saturnian ring system the F ring is very narrow indeed. Interestingly, there may be structures of even smaller scale in the F ring. First, the IPP team found indication of clumpiness in the ring. Second, the charged particle absorption features (the electron data, in particular) displayed distinct variations between $2.34 R_s$ and $2.36 R_s$ (refs 4, 5). These structures, if reflecting the condensation of matter around the F ring, would mean the presence of one or more rings (or satellites) with dimensions between 100 and 200 km.

Clearly, the particulate distributions are basic to the understanding of the F ring structure and the charged particle absorption taking place there. While some considerations on the nature of the F ring have been given by the Pioneer 11 experimenters^{4,5,7}, and by Dermott *et al.*⁸ in connection with their theoretical study of the narrow rings of Jupiter and Uranus, a more detailed ring model will be constructed here taking advantage of the published IPP and charged particle results. As

planetary ring absorption is likely to play an important part in the budget of trapped radiation in the magnetospheres of Jupiter and Uranus as well as that of Saturn, we take the F ring as an example to outline some of the essential points of interaction between the planetary rings and the magnetospheric plasma. In particular we will explore the possibility of deducing the physical nature of the planetary rings from a combination of the optical and charged particle observations.

The most interesting F ring absorption features concern the sharp minima at 2.340 and $2.355 R_s$ observed in both of the inbound and outbound passes together with a central dip in the electron flux of 10 MeV energy range, which was detected only in the inbound pass^{4,5}. Briefly, such absorption structures could be produced by three different (idealized) combinations of the ring system:

- (1) One single circular ring in a dipole field with certain equatorial offset ($\Delta r \approx 0.01 R_s$);
- (2) One single elliptical ring in a dipole field with negligible equatorial offset ($\Delta r \ll 0.01 R_s$);
- (3) Two circular rings in a dipole field, again, with negligible equatorial offset.

The first two cases are essentially similar and the actual situation may be intermediate between them (that is, one single elliptical ring in a dipole field slightly offset with $\Delta r \leq 0.01 R_s$, see Dermott *et al.*⁸) and this class of ring models will be illustrated by considering case (2). As shown in Fig. 1, charged particles drifting at different L shells would traverse different lengths of the ring material and hence experience varying degrees of reduction in particle flux⁹. As a result of such a longitudinal sampling effect, a radial profile of the absorption feature would be quite non-uniform with absorption minima formed at the inner and outer edges of the sweeping corridor of the ring. If we assume that the absorption profiles (such as locations and widths of the absorption dips) should have a certain correspondence with the radial variation of the integrated mass density or effective optical thickness, some physical parameters of the F ring may be derived. For instance, a plausible choice in which case the ring particles are bound by two orbits with $a_1 = 2.3500 R_s$, $a_2 = 2.3479 R_s$, $e_1 = 0.00468$, $e_2 = 0.00415$, and that the lines of apsides of these two orbits are aligned, could give a reasonable fit to the Chicago data⁴. Note that the maximum width of the F ring is about 200 km and the minimum only 50 km here, while the absorption effect extends over a region of 1,400 km. Such a ring configuration resembles the ϵ ring of Uranus¹⁰⁻¹³, and by the same token this narrow ring is most likely maintained by a small satellite⁸. Whilst the double absorption minima flanking the ring are caused by the effect of longitudinal sampling of the charged particles, a sharp drop in the particle flux due to local absorption would also be observed if the spacecraft is situated in the near vicinity of the satellite or the main body of the ring (as may be the case for the inbound pass).

Some estimate for the optical thickness of the absorbing particles may be obtained by examining the two absorption minima. As outlined before^{7,14-16}, the cumulative reduction effect as the charged particles diffuse inward across a radial distance ΔL can be expressed by the survival probability given as:

$$P = \exp(-2N_b\tau) \quad (1)$$

Where N_b is the total number of bounces executed by the charged particles and τ the effective optical depth of the absorbing particles with diameter larger than the absorption range R (~ 5 cm). Defining the radial diffusion time scale as:

$$t_d = \frac{(\Delta L)^2}{4D} \quad (2)$$

(D is the radial diffusion coefficient), and the bounce period t_b is taken to be 2.4 s as appropriate for the 10 MeV electrons. Then with the relation $N_b = t_d/t_b$ plus using $\Delta L \approx 200$ km we find

$\tau \leq 3.4 \times 10^6 D$ as $P \approx 5 \times 10^{-2}$ at the outer absorption minimum observed in the outbound pass⁴. Since no more than 20% of the ring material will be sampled in one drift the actual relation should read: $\tau \leq 0.8 \times 10^7 D$.

Only upper limits of D have been given [$D < 10^{-8} R_s^2 s^{-1}$ at $3.1 R_s$, or $D < 6 \times 10^{-7} R_s^2 s^{-1}$ at $3.1 R_s$ and $10^{-7} R_s^2 s^{-1}$ at $2.5 R_s$ (ref. 7)]. Consider that the radial diffusion coefficient in the saturnian magnetosphere might have a very steep L -dependence as in the case of the Earth's magnetosphere ($D \propto L^{10}$, see ref. 17), it is possible that $D \leq 10^{-9} R_s^2 s^{-1}$ at the position of the F ring; thus $\tau \leq 0.8 \times 10^{-2}$. This value is compatible with the value of $> 5 \times 10^{-5}$ as derived from using a somewhat different method⁷, and with the IPP result of $\leq 0.8 \times 10^{-2}$ if the width of the ring is taken to be 200 km.

Although these values appear to be in satisfactory agreement, the diffusion time scale (t_d) as determined by equation (2) is probably too short in comparison with the drift period of the 7–17 MeV electrons in question (that is, 3×10^3 s compared with 7×10^4 s). In other words, two narrow rings with complete coverage at all longitudes [that is case (3)] might be required instead of just one with partial longitudinal coverage which allows rapid refilling of the absorption dips. To this binary ring

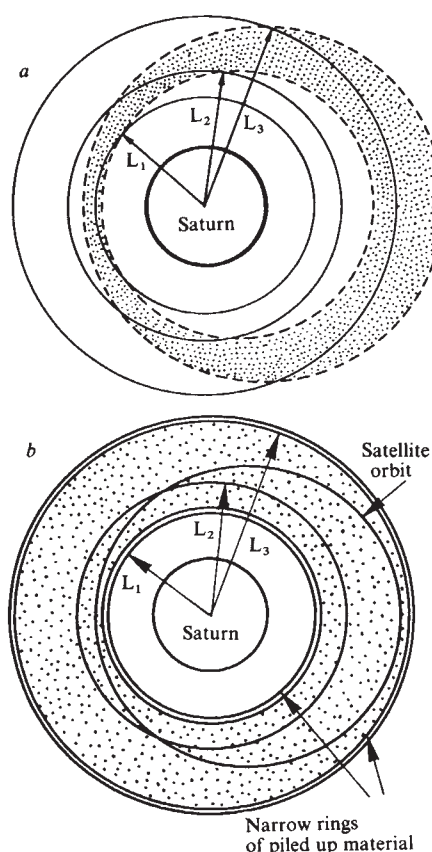


Fig. 1 Sketch of two possible ring configurations to interpret the observed charged particle absorption effects. *a*, One single ring with certain eccentricity and varying width. The charged particles drifting in different L shells would penetrate through different mass of ring material resulting in the formation of sharp absorption minima near perihelion and aphelion. If the diffusion time scale across the ring width is too short the absorption dips due to such longitudinal sampling effect could be refilled at certain positions. *b*, Two circular rings flanking the orbit of a small satellite in elliptical orbit. Two absorption minima would always be imprinted at all longitudes in the charged particle fluxes. If the spacecraft passes directly above or below the ring or satellite, an absorption spike would be observed as well as the inner and outer dips. Also the dipole offset, assumed to be zero here, could actually have a value like a fraction of $0.1 R_s$.

system we should add another satellite orbiting between them with semimajor axis $a \approx 2.35 R_s$ and eccentricity $e \approx 0.004$.

The physical basis for the formation of such a system may be depicted as follows. After the fragments are ejected from the satellite surface at low speeds as a result of meteoritic bombardment or tidal stress, they would first follow trajectories approximating that of the parent satellite. However, they will be gradually cleared away within the sweeping corridor of the satellite, because of recapture by the satellite, or inward and outward diffusion due to inelastic collision with the satellite or with other small particles. The process of orbital dispersion, in any case, cannot continue forever as it is limited by the collisional frequency and mass density distribution of the interacting medium. It can be easily seen then there will be material piled up at the regions just bordering with the perihelion and aphelion of the satellite. As the particles injected into these edges would all have near circular orbits as a result of prior inelastic collisions, no further orbital dispersion could occur¹⁸; in this way two narrow rings of dense matter would be formed confining a wider zone of more diffuse nature. Note that such an orbital equilibrium configuration has been discussed before by Hénon¹⁹ but in a somewhat different context.

A clear choice of the F ring configuration is difficult, but the possibility of a binary ring system is emphasized here as it has the potential of shedding new light on the structures of the rings of Uranus and Jupiter.

Another apparent puzzle in the Pioneer data may have some implication on the structure of the F ring—there is no signature of absorption of charged particles at the central position ($2.33 R_s$) of the F ring as identified by the IPP team. Barring systematic error in the spacecraft position by a margin of 0.01 – $0.02 R_s$ for this moment, we would have to conclude that the F ring detected by the imaging experiment was not responsible for the charged particle absorption. This has several interesting implications about the F ring system.

First, in order to reduce its absorption power but amplify its cross section for light reflection the optical component of the F ring must consist mainly of tiny dust particles of submillimetre size if not smaller. To check this point let us note that the survival probability for charged particles diffusing across a belt of dust particles with average diameter $d \ll R$ can be written as:

$$P = \exp(-2N_b X/R) \quad (3)$$

where $X = 2d\tau/3$. Now, with $P \geq 0.95$ as judged from the electron data^{4,5,7} we have immediately $d \leq 5.2 \times 10^{-3}$ cm if the ring width ΔL is about 800 km and $d \leq 2.1 \times 10^{-2}$ cm if $\Delta L \approx 200$ km. In the above calculation we have assumed $D \approx 10^{-9} R_s^2 s^{-1}$ and $\tau \approx 2 \times 10^{-3} (800/\Delta L)$. If the albedo α of these particles is smaller than unity d would be even smaller. In the extreme case of $\alpha \approx 0.05$, as was found for the uranian ring particles^{20,21}, we have $d \leq 3 \times 10^{-4}$ – 10^{-3} cm. From this point of view, the F ring identified by the IPP experiment may be in fact a dust belt of micrometre-sized particles similar to the jovian ring^{22,23}.

Second, to boost the absorption power but limit the cross section for light reflection to a small value the ring particles between $2.34 R_s$ and $2.36 R_s$ must be of centimetre size if not larger. Also, to explain the absence of this ring in the optical observation a low albedo must be invoked. In this way an optical thickness of $\approx 3 \times 10^{-3}$ (for $\alpha \approx 0(1)$) as required to interpret the absorption feature would be reduced to $\leq 2 \times 10^{-4}$ if $\alpha \approx 0.05$. Such a value of optical depth may be below the limit of optical detection. On dynamical grounds, particles of metre size or so are required to maintain the stability of the narrow ring configuration against Poynting-Robertson effect²⁴. But is there reason for such a low albedo? Presumably so, as whatever volatile ices on the particle surface would be sputtered away by the energetic ions exposing the (carbonaceous chondritic?) core material of low albedo²⁵. [A similar thing can be said of the particles at the outer edge of the A ring in which case there is a difference of about $0.02 R_s$ between the IPP result and the charged particle data³⁻⁷.]

Finally, that the dust belt of micrometre-sized particles should be located inside of the narrow ring of larger particles is consistent with its being of fragmentation origin—as proposed for the jovian ring^{26–29}. Note that the rings of both Jupiter and Uranus have been suggested to be the fragments of small satellites which are spiralling inwards towards the central planets. According to Dermott *et al.*³⁰, for instance, the reason for such orbital decay is basically that the synchronous orbits of both Jupiter and Uranus are outside the Roche limits, so satellites formed between these two positions would be pulled inward as a result of planetary tidal action. For Saturn, the synchronous orbit at $1.8 R_s$ is inside of the Roche limit at $2.3 R_s$, so a reverse process might take place there—with small satellites accreted from condensations in the A ring being gradually pushed away from the planet. The F ring and its parent satellite are then manifestations of just such a process²⁶.

We have discussed here the possibility that the F ring may have two components: (1) the inner one mainly consisting of micrometre-sized dust particles (detected by the IPP experiment), and (2) the outer one of centimetre- to metre-sized bodies emitted from a small satellite (observed by the charged particle detectors). This scenario makes interesting comparison with the discovery of the ring system of Jupiter^{22,23,31,32}. We have also investigated the possibility of deducing the ring configuration capitalizing on the radial profiles of the observed absorption features of the charged particles. Besides the more conventional configuration of one single narrow ring we find that a double-ring system is also a possibility—if not strictly required by the particle data. Detailed intercomparison of the Pioneer IPP, particles and fields observations may allow us to narrow down some of the uncertainties. Also, the Voyager I flyby in November, which follows essentially the same trajectory, should provide another set of valuable data. From analysis of charged particle experimental data, Simpson *et al.*³³ have concluded that the symmetric pair of the F ring absorption dips observed both inbound and outbound, is caused by two separate narrow rings; Gehrels³⁴ has found that the high colour index of the optical F ring is consistent with the idea that it is made of micrometre-sized dust particles.

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Order-modulated structures and the thermodynamics of cordierite reactions

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The determination of palaeo pressures and temperatures (P , T) in metamorphic belts is of fundamental importance to our understanding of large-scale physical processes in the Earth. Such determinations are made by comparing observed mineral assemblages with experimental data on their P , T stability fields¹, thus assuming that the experiments are an adequate model for the natural reaction. The characterization of the structural state of natural and experimental materials is particularly important in this context. This note demonstrates the effect of the state of cation order on the P , T slopes of metamorphic reactions involving cordierite, $(\text{Mg}, \text{Fe})_2\text{Al}_4\text{Si}_2\text{O}_{18}$, an indicator phase in metamorphism. Particular emphasis will be placed on the mechanism of Al, Si ordering in cordierite in so far as it relates to the characterization of the degree of cation order and hence the configurational entropy contribution to the thermodynamics of cordierite reactions.

Mg-cordierite occurs in two polymorphic forms. In the high temperature hexagonal structure, stable above $\sim 1,450^\circ\text{C}$ (refs 2, 3), the Al and Si atoms are distributed over two sets of tetrahedral sites—three T_1 and six T_2 sites per formula unit $\text{Mg}_2\text{Al}_4\text{Si}_2\text{O}_{18}$ (Fig. 1). The Al, Si distribution involves two Al and one Si atom disordered over the T_1 sites and two Al and four Si atoms disordered over the T_2 sites⁴. In the slightly distorted low temperature orthorhombic form the T_1 sites are split into two non-equivalent sites T_{11} and T_{16} , while the T_2 sites are split into a set of three non-equivalent sites T_{21} , T_{26} and T_{23} . Within this new structure the Al, Si atoms are able to form a completely ordered distribution (see Table 1).

Experiments carried out on the mechanism and kinetics of the hexagonal–orthorhombic transition in anhydrous Mg-cordierite confirm that the polymorphism is due to Al, Si ordering and furthermore demonstrate that in equilibrium conditions the ordering transformation is first order involving the discontinuous transformation from a disordered to a well ordered phase at temperatures below $\sim 1,450^\circ\text{C}$ (refs 3, 5).

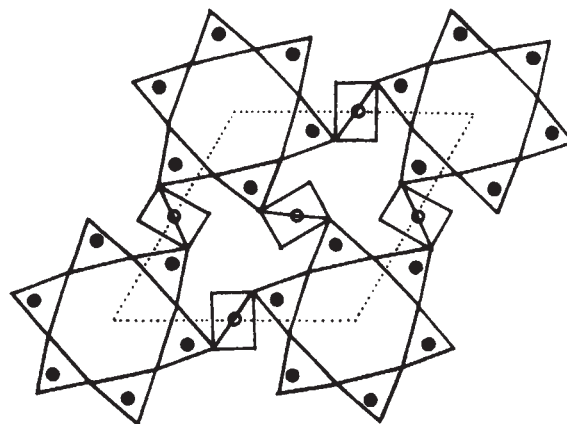


Fig. 1 The structure of hexagonal cordierite showing only the tetrahedral linkages. The tetrahedra in the ring sites (●) are designated T_2 , while those outside the ring (○) are designated T_1 in Table 1. The dotted line is the hexagonal unit cell.

This polymorphism has been generally characterized by a distortion index Δ , based on the degree of splitting of the peaks derived from the 211 hexagonal peak in an X-ray diffractometer trace⁶. In the hexagonal form Δ is zero, while in the fully ordered orthorhombic form of anhydrous Mg cordierite $\Delta \approx 0.25^\circ$. In nature, however, cordierites of intermediate Δ values are common⁷ and although various compositional effects have been proposed to account for this⁸⁻¹⁰, the occurrence of these intermediate states in anhydrous Mg-cordierite² has yet to be explained. The strongly first order nature of the transformation precludes the formation, in equilibrium conditions, of intermediate structural states. As intermediate cordierites are formed from hexagonal cordierite at temperatures substantially below the equilibrium ordering temperature (that is, in the stability field of fully ordered cordierite), it is pertinent to examine the mechanism of Al, Si ordering in non-equilibrium conditions.

Isothermal annealing experiments were carried out on stoichiometric Mg-cordierite glass at temperatures between 1,150 and 1,430 °C for periods of 0.5–400 h. The preparation and characterization of the starting material and experimental details are given elsewhere³. The products of the annealing runs were studied by X-ray diffractometry and transmission electron microscopy.

The first phase to crystallize from the glass is invariably homogeneous hexagonal cordierite, which eventually transforms on annealing to homogeneous orthorhombic cordierite, the stable phase. The mechanism of the transformation in metastable conditions, however, does not involve the direct nucleation and growth of the orthorhombic phase. The transformation proceeds by the formation of a modulated structure in which two orthogonal distortion waves produce a 'tweed' microstructure in transmission electron micrographs (Fig. 2). The average structure as observed by X-ray diffractometry is hexagonal, whereas locally the structure consists of a sinusoidal variation in the degree of distortion towards two twin-related orthorhombic variants. The overall structure may be described as order-modulated, in which the short-range order may be high, while the long-range order, as measured by X-ray diffraction, is zero.

Continued annealing results in a gradual overall coarsening of the microstructure although the unequal development of one or other variant may lead to regions in which the distortions become unbalanced. This manifests itself as a slight departure from the average hexagonal state, and an apparently continuous range of Δ values is obtained by the increasing heterogeneity and growth of the microstructure. The first signs of peak splitting in the X-ray diffractometer traces must already be equated with a considerable degree of coarsening of the microstructure, on a scale of thousands of angstroms. Full details of the coarsening mechanisms will be published elsewhere, but there are two principal conclusions which can be drawn from these results. First, a mechanism which involves the formation of a metastable order-modulated structure will not be detected by methods such as X-ray powder diffractometry which measure long-range effects, and hence any assessment of the structural state obtained by such methods may be in error. Second, the mechanism described here is quite different in character from

Table 2 Volumes and entropies of minerals at 700 °C and 1 atm (ref. 12)

	Volume (cm ³ gfw)	Entropy (cal °C gfw)
Cordierite 2MgO . 2Al ₂ O ₃ . 5SiO ₂ gfw = 584.97	234.01	264.58
Sapphirine 2MgO . 2Al ₂ O ₃ . SiO ₂ gfw = 344.63	100.55	151.38
Quartz SiO ₂ gfw = 60.085	23.72	27.34

the temperature-dependent ordering models used by workers currently dealing with the thermodynamics of cordierite reactions¹¹⁻¹³.

One major assumption in the experimental determination of the P, T stability field of anhydrous cordierite has been that the configurational entropy contribution of Al, Si order is not large^{12,13}. This assumption is based on several lines of evidence all of which depend on an interpretation of the limited data provided by X-ray powder diffraction. First, measured heats of solution of Mg-cordierite with Δ values of 0.10, 0.20 and 0.25 prepared in metastable conditions are not significantly different^{11,12}. As Al, Si disorder is normally accompanied by considerable enthalpy effects, the conclusion is that all of the samples were similar and had a high degree of order. While this conclusion is in itself valid, it is interpreted in terms of a model in which the degree of order is assumed to be related to a continuously changing Δ value with temperature. Hence the observation that a considerable change in Δ value corresponds to a small inferred change in cation distribution is extrapolated to suggest that hexagonal cordierite with a Δ value of zero must have a high degree of Al, Si order.

However, the order-modulated mechanism described here suggests a different interpretation of the same observations. Ordering via an order-modulated structure results in a high degree of short-range order without any change in symmetry. Thus calorimetric measurements of order-modulated material would be expected to produce results very similar to that of fully ordered orthorhombic cordierite, and subsequent increases in Δ on annealing would have little further enthalpy effects. However, the essentially discontinuous nature of the ordering processes on a local level in the transition from homogeneous hexagonal cordierite (that is disordered) to order-modulated hexagonal cordierite does not allow extrapolation of intermediate Δ values to zero.

Conventional single crystal X-ray structure analysis is not able to distinguish between a completely disordered hexagonal cordierite (Table 1) and the order-modulated state described here, and although the Bragg-Williams order parameter seems to be the same in each case⁴, the configurational entropy of the two states may be radically different, as discussed below.

The extent to which the configurational entropy contribution to the total entropy change in a metamorphic reaction affects the P, T slope may be calculated from the Clapeyron relation $dP/dT = \Delta S/\Delta V$. Although the volumetric effects of cation disorder are negligible, the configurational entropy may be the dominant term, particularly in solid-solid reactions where the overall entropy changes are small. This is well illustrated by the reaction:

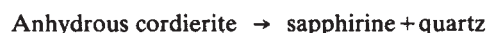


Table 2 shows the volume and third law entropy data from which dP/dT may be calculated. To evaluate the effect of configurational entropy of cordierite alone, the configurational entropy of sapphirine will be taken to be fixed at +4.2 cal per °C gfw as suggested by Newton *et al.*¹². The validity of this assumption will be discussed below.

Table 1 Tetrahedral sites in the cordierite polymorphs

Nine sites per formula unit Mg ₂ Al ₄ Si ₅ O ₁₈			
Hexagonal form	Orthorhombic form	Site content	
3 T_1 sites	2 T_{11} sites	2 Al	
	1 T_{16} site	1 Si	
	2 T_{21} sites	2 Si	
6 T_2 sites	2 T_{23} sites	2 Si	
	2 T_{26} sites	2 Al	

The entropy change for the reaction involving fully ordered cordierite ($S_{\text{config.}} = 0$) is therefore given by tabulated third law entropies:

$$\begin{aligned}\Delta S_{\text{reaction}} &= S_{\text{sapph}} + 4S_{\text{qtz}} - S_{\text{cord}} \\ &= (151.38 + 4.2) + (4 \times 27.34) - 264.58 \\ &= 0.32 \text{ cal per } ^\circ\text{C gfw} \\ \Delta V_{\text{reaction}} &= -38.3 \text{ cm}^3 = -0.915 \text{ cal bar gfw}\end{aligned}$$

Therefore

$$\frac{dP}{dT} = -0.35 \text{ bar per } ^\circ\text{C}$$

To calculate the slope for this reaction when the cordierite is completely disordered, the configurational entropy of the cordierite must be evaluated. Taking four Al and five Si atoms disordered over the nine T_1 , T_2 sites taken together,

$$\begin{aligned}S_{\text{config.}} &= -9R \left(\frac{4}{9} \ln \frac{4}{9} + \frac{5}{9} \ln \frac{5}{9} \right) \\ &= 12.0 \text{ cal per } ^\circ\text{C gfw}\end{aligned}$$

For the hexagonal structure described in Table 1 with two Al and one Si atoms disordered over T_1 sites and two Al and four Si atoms disordered over T_2 sites

$$\begin{aligned}S_{\text{config.}} &= -3R \left(\frac{2}{3} \ln \frac{2}{3} + \frac{1}{3} \ln \frac{1}{3} \right) - 6R \left(\frac{2}{6} \ln \frac{2}{6} + \frac{4}{6} \ln \frac{4}{6} \right) \\ &= 11.34 \text{ cal per } ^\circ\text{C gfw}\end{aligned}$$

Thus, although the Bragg-Williams order parameter of this state⁴ may suggest that it is partially ordered, its configurational entropy is very high. In contrast, an order-modulated state with the same apparent long-range order parameter would have a very small configurational entropy.

The dP/dT for the reaction involving hexagonal disordered cordierite with the cation distribution shown in Table 1 is

$$\frac{dP}{dT} = \frac{0.32 - 11.34}{-0.915} = 12 \text{ bar per } ^\circ\text{C}$$

The P , T slopes for the breakdown of ordered and disordered cordierite to sapphirine + quartz are shown in Fig. 3. The lines necessarily intersect at the order-disorder transition temperature (1,450 °C). The position of this intersection on the pressure axis is defined by reversed experiments and calorimetric data^{12,14} in which the equilibrium for the reaction using well ordered cordierite ($S_{\text{config.}}$ assumed to be 1.22 cal per °C gfw) was found to occur in the range 8.2 ± 0.4 kbar at 1,300–1,400 °C with a slope of about 1 bar per °C. This reaction line (dotted in Fig. 3) must also pass through the order-disorder transition temperature for Mg-cordierite.

Thus, while Newton's calorimetric and experimental data are internally self consistent, they represent only one solution for a cordierite with a particular state of order. The fact that the cation disorder in cordierite cannot be dismissed as insignificant stresses the importance of carrying out thermochemical measurements, and experimental high P , high T reactions, on materials in which the state of order has been characterized. Routine transmission electron microscopy combined with X-ray diffraction of such materials can provide considerably more information on short-range order as well as on the mechanism of the ordering process. This latter point is particularly important in relation to the estimation of the relevant configurational entropy corrections.

In the above treatment the effect of disorder in cordierite has been evaluated by assuming a fixed entropy for sapphirine.

However, the situation with regard to assumptions of cation order in sapphirine poses even greater problems than in cordierite. The occurrence of two polymorphs and their intimate intergrowths^{15,16}, and the possibility of a relationship between the polymorphism and Al, Mg and/or Al, Si disorder suggests the need for considerable caution over estimates of configurational entropy. Further, calorimetric measurements on synthetic and natural sapphirines¹⁷ disclose differences which have been attributed to different structural states; hence the synthetic material is not a good model for its natural counterpart. The sensitivity of P , T slopes of dry reactions to configurational entropy corrections, coupled with the structural variations present in natural sapphirines, further underlines the inadequacy of assumptions of $S_{\text{config.}}$ made on the basis of a single X-ray structure analysis of a sample.

Finally, it remains to consider the relevance of a discussion of disordered phases in experimental reactions to natural assemblages. The crystallization of a disordered phase within the stability field of the ordered polymorph is frequently observed where the starting material is in a highly metastable state, such as a glass. This may be a readily understood in terms of the large overall free energy change in the reactions involved with the formation of either phase, in which case the discrimination in favour of the metastable disordered phase is on kinetic grounds, its crystallization having a higher entropy of activation.

In a metamorphic rock, conditions are generally nearer to equilibrium. Consider the prograde formation of a phase with ordered and disordered polymorphs at a temperature below its order-disorder temperature. At constant pressure, the equilibrium temperature (T_1) for any reaction producing the ordered phase will necessarily be lower than the metastable equilibrium temperature (T_2) for the same reaction producing the disordered phase. Above T_2 the enhanced kinetics would favour the nucleation of the disordered phase. Thus, the formation of disordered phases in prograde reactions requires a measure of overstepping of the stable equilibrium temperature, a nearly universal phenomenon in dry rocks.

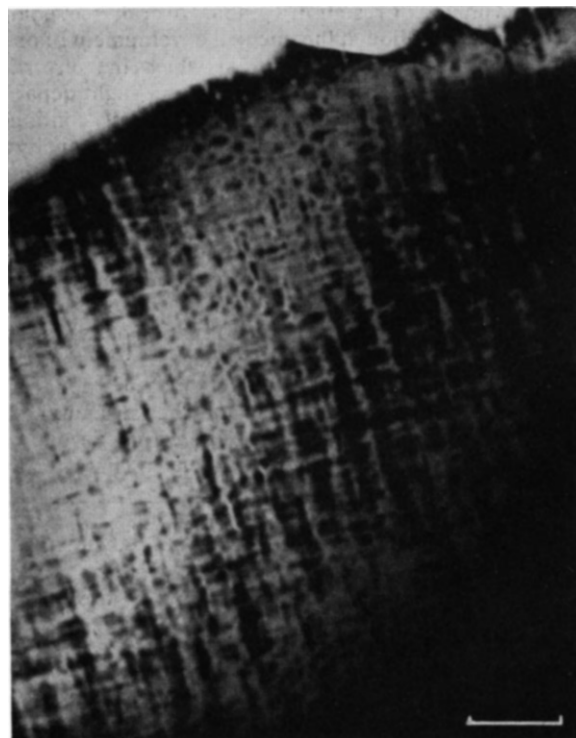


Fig. 2 Transmission electron micrograph of the order-modulated microstructure of cordierite, showing two orthogonal distortion waves normal to (100) and (120) planes of the hexagonal cell. Scale bar, 0.2 μm .

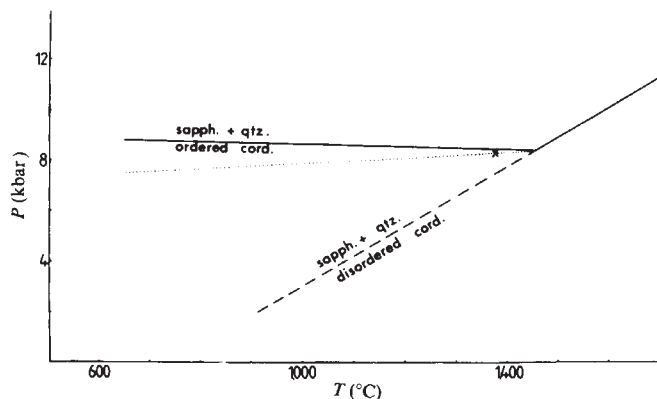


Fig. 3 Pressure-temperature equilibria for the reaction cordierite $\rightarrow$ sapphirine + quartz for ordered and disordered cordierite. The dotted line and the point X represent the slope and experimental point, respectively, determined for this reaction by Newton *et al.*¹² using a cordierite with assumed $S_{\text{config.}}$ of 1.22 cal $^{\circ}\text{C}$ gfw.

The extent to which disorder plays a major role in the growth of a mineral during metamorphism must be assessed for each individual occurrence. Furthermore, the structural state of a mineral exposed at the Earth's surface is not necessarily the same as that during its growth, and depends on the relative kinetics of the mineral reaction and the ordering process. In this respect the relict microstructure may indicate whether an ordered phase was nucleated directly or has been produced from a disordered phase subsequent to growth. The formation of intermediate structural states in cordierite strongly suggests that ordering occurs via an order-modulated structure.

The microstructures of natural cordierites have not yet been studied. However, in an investigation of the distortion indices of 128 natural cordierites⁷, the whole range of Δ values from 0 to >0.25 has been found, and these show characteristic frequency distributions for various geological environments. Notwithstanding the possible compositional effects on Δ values⁸⁻¹⁰, this supports Schreyer's hypothesis that the observed Δ values indicate an evolution from an initially disordered high cordierite⁷ similar to the process in the experiments described here.

Clearly many of these implications to the P, T slopes of natural metamorphic reactions remain to be tested. The evidence suggests the need for calorimetric measurements on phases with well characterized microstructures, for repeating experiments on metamorphic reactions using both ordered and disordered reactants, and finally for the characterization of the structural states of the natural assemblages to which experimental studies are applied.

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D.c. electrical conductivity of Green River oil shales

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The recent interest in the kinetics and mechanistic aspects of the thermal decomposition of oil shale kerogen¹ derives largely from its importance as an alternative energy source to petroleum. In spite of considerable research effort, how oil shale kerogen decomposes to gaseous and liquid hydrocarbon fuels is still not fully understood¹. The present study considers the d.c. electrical conduction behaviour of Green River oil shales. The observed trends in the electrical behaviour of these materials are correlated with a two-step decomposition model in which the rate-determining processes are shown to be (1) breakdown of an outershell polar bridge structure with an activation energy of 15 ± 2 kcal mol^{-1} (180-350 $^{\circ}\text{C}$) and (2) cleavage of an inner core naphthenic structure also involving polar groups with an activation energy of 35 ± 3 kcal mol^{-1} (350-500 $^{\circ}\text{C}$). These structural changes are shown to correspond to the chemical transformation of kerogen to liquid and gaseous hydrocarbons through a bitumen intermediate. Although the present data pertain to Green River oil shales in particular, the observed trend of charge transfer mechanisms in the thermal decomposition behaviour of thermally unstable materials may be indicative of how thermal and electrical properties of all solid materials in general are closely coupled. The common mechanistic origin identified in the present study for the processes of thermal decomposition and electrical conduction, offers the possibility of improving the thermal stability characteristics of a solid by appropriate changes in the electric field surrounding it.

Electrical conductivity (σ) measurements were carried out in the temperature range 25-500 $^{\circ}\text{C}$, on right circular cylindrical disks of oil shales of diameter ~ 2.4 cm and thickness of 0.25 cm. The direction of the electric field was perpendicular to the shale stratigraphic planes in all the cases reported below. A nominal field strength of 110 V cm^{-1} was applied across the shale sample and the currents were measured using a conventional operational amplifier circuit. Silver electrodes were used for the measurements; the opposite faces of the sample disks were also coated with silver paint to minimize contact resistance. In a typical 'run', the sample was heated in an atmosphere of dry pre-purified N_2 at a heating rate of ~ 2 $^{\circ}\text{C}$ min^{-1} . The required d.c. voltage was then imposed across the shale sample. Because of polarization effects, the currents slowly decayed with time at a given temperature—the values finally attaining a steady-state. This steady-state conductivity σ_{∞} was recorded at various temperatures and the data displayed in the form of conventional $\ln \sigma_{\infty}$ versus $1/T$ plots².

Figure 1 shows the variation of d.c. conductivity with temperature for a Utah shale, a Colorado oil shale sample and a Wyoming shale³. The organic content of these samples was estimated from specific gravity measurements⁴ and is expressed in terms of an equivalent Fischer assay as the volume of oil in litres that can be extracted per unit mass (tonne) of the shale. The σT versus $1/T$ plots can be broken up into approximately three segments with activation energies (slopes) E_1 , E_2 , and E_3 respectively. These values for the different samples and the corresponding temperature intervals are shown in Table 1. The E values within each temperature interval are seen to be comparable for the different samples within the limits of experimental error. More significantly, the slopes are also seen to be relatively insensitive to the amount of organic matter in the shale (Fig. 2 and Table 1). This evidence strongly supports the

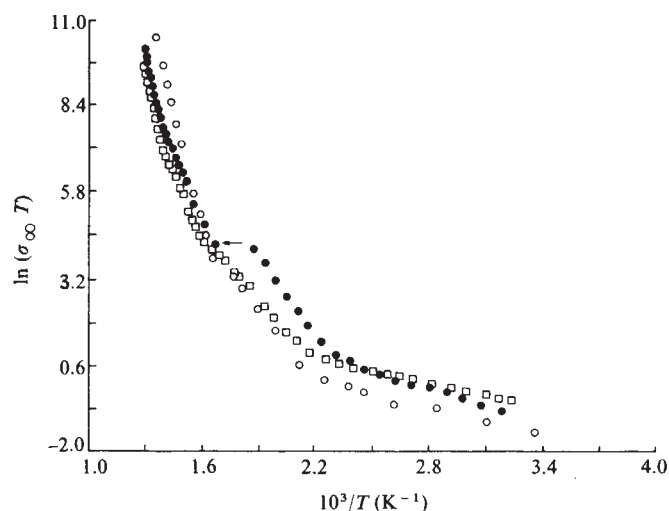


Fig. 1 Variation of steady-state conductivity with temperature for Green River oil shales: □, Wyoming oil shale (135 l tonne⁻¹); ○, Utah oil shale (344 l tonne⁻¹); ●, Colorado oil shale (140 l tonne⁻¹). The arrow denotes a set of isothermal conditions for approximately 10 min followed by resumption of the heating program (2 °C min⁻¹, see text).

linking of the observed E values to an intrinsic charge transfer process whose energetics are independent of the amount of organic matter in the shale. A dependence of the E values on the organic content of the shale would have precluded a straightforward explanation of the observed trends in terms of charge transfer processes. Chemical effects such as decomposition are expected to bring about changes in the magnitude of E with organic content in such a case. The magnitude of the measured σ values, however, do show pronounced time-dependent effects, especially at elevated temperatures (>400 °C, compare with the values denoted by arrows in Figs 1 and 2). These irreversible effects are attributed to thermal decomposition of the indigenous organic matter in the shale. Further evidence is the pronounced hysteresis in σ_{∞} values that are observed after heating to ~500 °C and cooling back to room temperature. The σT versus $1/T$ plots show completely different activation behaviour in the cooling cycle. A second heating does not significantly bring about further changes and the conductivity behaviour now reflects charge migration in the residual carbon and in the inorganic mineral matrix in the shale.

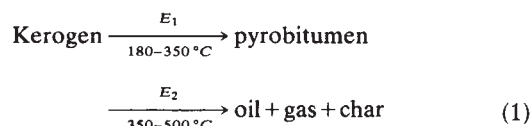
Table 1 Activation energies* obtained from d.c. electrical conductivity measurements on Green River oil shales

Sample (oil yield) (l tonne ⁻¹)	Activation energy† (kcal mol ⁻¹) (approximate temperature interval)
Utah oil shale (344)	$E_1 = 38$ (350–500 °C) $E_2 = 15$ (180–350 °C) $E_3 = 3$ (25–180 °C)
Wyoming oil shale (135) H	$E_1 = 36$ (350–500 °C) $E_2 = 14$ (180–350 °C) $E_3 = 3$ (25–180 °C)
Colorado oil shale (140)	$E_1 = 32$ (280–500 °C) $E_2 = 16$ (180–280 °C) $E_3 = 3$ (25–180 °C)
Colorado oil shale (244)	$E_1 = 34$ (350–500 °C) $E_2 = 17$ (180–350 °C) $E_3 = 2$ (25–180 °C)

* Measured values include contribution from migration enthalpies as well as carrier formation energies especially at elevated temperatures (see ref. 2).

† Nominal error in measured values, ± 2 kcal mol⁻¹.

The observed trends in the electrical conductivity behaviour are consistent with a two-step decomposition process for oil shale kerogen¹:



We propose that the two high-temperature regions in $\ln(\sigma_{\infty}T)$ versus $1/T$ plots (Figs 1 and 2) refer to the above reactions with activation energies E_1 and E_2 respectively. This interpretation is strongly supported by the good correlation of the present activation energies E_1 and E_2 with those measured recently in this laboratory for the thermal decomposition of Green River oil shale kerogen⁵. Using a non-isothermal thermogravimetry technique, activation energies of 14.53 kcal mol⁻¹ and 36.49 kcal mol⁻¹ were obtained for the low-temperature thermal decomposition of pyrobitumen and the high-temperature decomposition of kerogen respectively⁵. These values are in excellent agreement with those for E_1 and E_2 in Table 1. The close correspondence of activation energies obtained from two widely disparate techniques—one electrical and the other thermal—underlines a common origin for the processes of charge carrier migration and thermal decomposition in Green River oil shales. As only charged species contribute to the d.c. conductivity, the primary bond-breaking step must involve polar molecules in both stages of reaction (1). A previous structural model^{6,7} for oil shale kerogen, involves a non-uniform three-dimensional network wherein the major components (such as isoprenoids, steroids, and terpenoids) are linked by

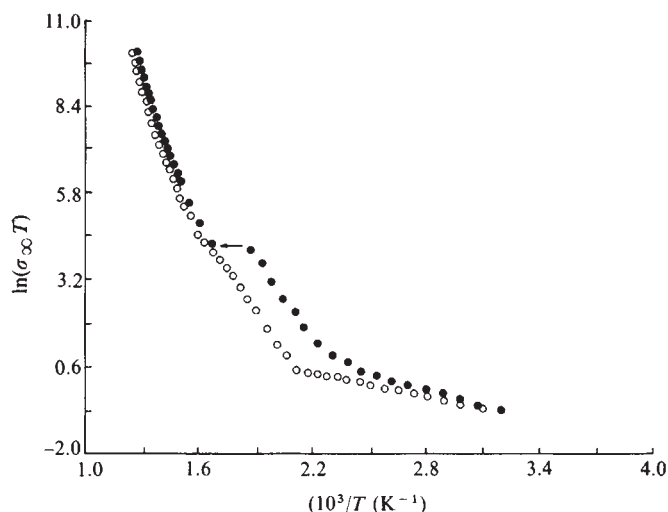


Fig. 2 Comparison of d.c. electrical conductivity behaviour for Anvil Points (Colorado) oil shales containing two different levels of organic content: ●, 140 l tonne⁻¹ (same sample as in Fig. 1); ○, 240 l tonne⁻¹.

bridges to form the kerogen matrix. Based on the present data, we propose that rupture of cross-linking bridges, most of which are polar in nature (disulphides, ethers and ester groups), is the rate-determining step in both pyrolysis as well as in charge migration. Two types of polar bridges are envisaged: (1) the first 'outershell' group which interlinks the major components and is broken first in the first stage of decomposition and (2) a second 'core' type which holds together the carbon skeletal chain (predominantly naphthenic) within each individual component and is ruptured in the second stage of pyrolysis⁷. The proposed mechanism is consistent with the increased solubility observed for kerogen at the end of the first pyrolysis step; the reduced

degree of cross-linking as a result of the breakdown in the bridge structure is expected to enhance the solubility of kerogen markedly.

The conductivity in the low-temperature region is expected to arise mostly from the indigenous moisture in the shale. This process is assigned an activation energy, E_3 (compare with Table 1). The water molecules are presumably trapped within the kerogen macromolecular network. The extremely low E_3 values observed at low temperatures imply a chain mechanism for conduction presumably of the Grotthus-type⁸.

Finally, although the present conductivity data have highlighted the importance of charge transfer in the thermal decomposition of oil shale kerogen, further measurements are necessary to elucidate the exact nature of these charged species. Note that similar conductivity measurements on a variety of solid fuels (such as coals) could yield a unified picture of the mechanistic aspects of charge migration and thermal instability in these materials.

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Late-Glacial history of the Brecon Beacons, South Wales

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In the uplands of the Brecon Beacons, Black Mountains, Fforest Fawr area of South Wales, small arcuate moraines and/or protalus ramparts are found below steep escarpments and in glacial cirques^{1–3}. The freshness and distinctive morphology of many of these features have led to the suggestion that they are not a product of the late Devensian ice sheet, but may have been formed during the Loch Lomond Stadial of the Late-Glacial period (Fig. 1), the last occasion when glaciers existed in the British Isles. I present here pollen-stratigraphic evidence and radiocarbon dates which form the basis for the first Late-Glacial chronology for this area of South Wales, and which prove that the features described above developed during the Loch Lomond Stadial (approximately equivalent with the Younger Dryas of Scandinavia^{4–6}).

Within the catchment of Glyn Tarell, on the northern side of the Brecon Beacons escarpment (Fig. 2), two distinctive end moraines occur. Beneath the north-east-facing scarp of Craig-y-Fro (grid ref. SO 972208), an arcuate ridge extends for about 0.75 km down the western slope and crosses the valley floor at ~365 m OD, while in the cirque of Craig Cerrig-gleisiad (grid ref. SO 964220), the innermost of a complex of glacial depositional landforms (some of which may be the product of the late Devensian ice sheet) is a prominent boulder-strewn, arcuate moraine at a height of ~425 m OD. Both moraines enclose infilled kettle-hole basins in which Flandrian limnic and terrestrial sediments have accumulated. The subsurface contours of the two basins were established by sounding the infills along a

Radiocarbon yr BP	The British Isles (adapted from ref. 31)		Godwin – Jessen pollen zones	Continental N.W. Europe (after ref.4)	Late Weichselian Late – Glacial		
10,000	Flandrian		IV	Flandrian			
	Loch Lomond Stadial		III	Younger Dryas			
11,000	Late Devensian Late – Glacial		II	Allerød			
				Older Dryas			
12,000			I	Bolling			
13,000				Middle Weichselian			
	Late Devensian						

Fig. 1 The Late-Glacial period.

grid pattern⁷ and the deepest points were located. In this way, the oldest sediments in the basins could be sampled. Overlapping cores were then removed with a piston corer for pollen analyses and radiocarbon dating.

At Traeth Mawr (grid ref. SO 967257), some 3.5 km north of Craig Cerrig-gleisiad (Fig. 2), a large dead-ice hollow at ~330 m OD contains a typical Late-Glacial sequence⁸ comprising organic muds between minerogenic sediments, and these deposits are, in turn, overlain by Flandrian muds and peats. Cores were removed from a deep part of the basin for pollen analyses, and samples for radiocarbon dating were obtained by bulking material from comparable stratigraphic horizons in six of the cores. As at the sites within the moraines, this procedure was made possible by the clearly defined stratigraphy of the basin sediments. The full stratigraphic sequences at the three sites, the pollen assemblage zones and the dated horizons are shown in Fig. 3. The radiocarbon dates are presented in Table 1.

Three dates were obtained from the Late-Glacial profile at Traeth Mawr, the earliest of which, 11,660 ± 140 BP (SRR-1562), was from the basal organic sediments. The pollen spectra from those horizons show marked upward trends in the curves for *Betula* and *Juniperus*, increases in aquatic taxa and declining values for open-habitat herbs such as *Artemisia* and *Rumex*. The radiocarbon date is, however, over 1,000 yr younger than age determinations from comparable Late-Glacial Interstadial deposits in Scotland^{9–12}, the Lake District^{13–15}, North Wales¹⁶ and Cornwall¹⁷. One reason for this is that initially low productivity within the lake during the early Late-Glacial Interstadial could have led to a very slow rate of sediment accumulation, and as deposition probably increased with the development of the lacustrine ecosystem, it would be extremely difficult to obtain a true age for the basal organic sediments from a 2-cm thick sample of material. This problem may have been exacerbated by the compaction of the unconsolidated organic sediments, a process that can result in several hundred years of sedimentary

Table 1 Radiocarbon dates from the Brecon Beacons

Sample no.	Site	Depth below surface (cm)	Date (yr BP)	$\delta^{13}\text{C}$ value
SRR-1560	Traeth Mawr	287–289	9970 ± 115	-16.9‰
SRR-1561	Traeth Mawr	369–371	10,620 ± 100	-17.8‰
SRR-1562	Traeth Mawr	397–399	11,660 ± 140	-16.4‰
SRR-1563	Craig-y-Fro	442–444	10,030 ± 100	-29.0‰
SRR-1564	Craig Cerrig-gleisiad	550–552	10,860 ± 70	-25.6‰

history becoming compressed into a few centimetres¹⁸. A third possibility is that the date has been affected by the incorporation of younger carbon, either during the sampling process or through sediment mixing on the former lake floor. Any of these factors, either singly or in combination, could have produced the date of $11,660 \pm 140$ BP which, in the context of other age determinations from Highland Britain, would seem to be anomalous.

The uppermost Interstadial deposits at Traeth Mawr yielded a date of $10,620 \pm 100$ BP (SRR-1561). At that level in the profile there is a marked decline in percentages of woody plant pollen, particularly *Betula* and *Juniperus*, the virtual disappearance of the thermophilous herb *Filipendula* cf. *ulmaria*, and a pronounced increase in percentages of taxa indicative of open habitats, notably *Rumex*, *Artemisia* and species of Caryophyllaceae and Cyperaceae. SRR-1561 agrees closely with the date of $10,590 \pm 185$ BP obtained from a similar biostratigraphic horizon at a site in south-east Ireland¹⁹ (although that assay was made on a 10-cm thick sample), but is markedly younger than the age of 11,000 BP assigned to the beginning of the Younger Dryas Chronozone in north-west Europe⁴. In Scotland and parts of northern England, the onset of cold climatic conditions and the build-up of Loch Lomond Advance ice seem to have begun before 11,000 BP (refs 5, 20), and thus the dates from Traeth Mawr and south-east Ireland, if correct, reflect the later onset of Stadial conditions and subsequent glacier development. This, in turn, might have been caused by time-transgressive southward movement of the oceanic Polar Front²¹, but additional dates are required from other areas of western Britain to test this hypothesis.

The end of the Loch Lomond Stadial at Traeth Mawr is dated at $9,970 \pm 115$ BP (SRR-1560). These early Flandrian horizons are characterized by high Gramineae frequencies, a rising *Juniperus* curve and low arboreal percentages. The radiocarbon date agrees well with dates of $10,080 \pm 320$ BP, $10,060 \pm 380$ BP and $10,170 \pm 220$ BP obtained from basal Flandrian sediments in infilled pingo basins in south Cardiganshire²²⁻²⁴, and a date of $10,200 \pm 220$ BP from the base of the Flandrian sequence in Tregaron Bog in west Wales²⁵, although the large standard

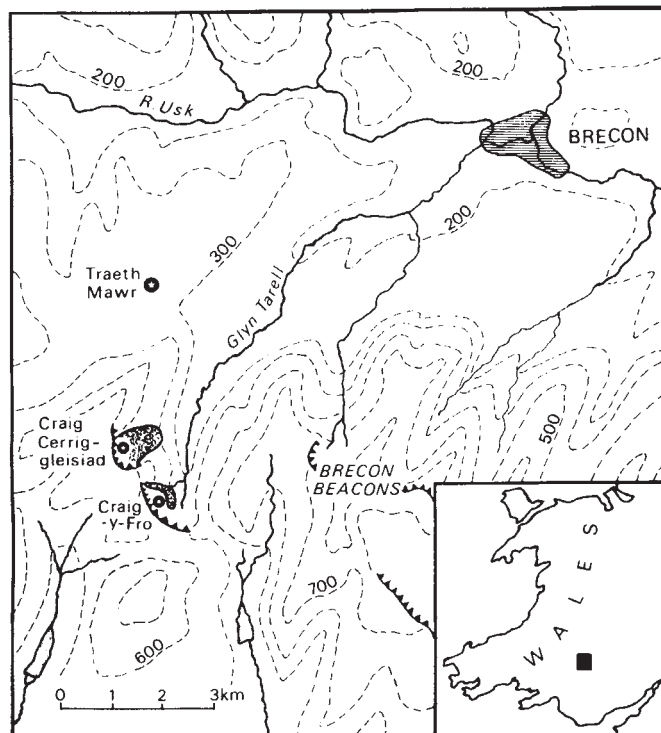


Fig. 3 Stratigraphies, pollen assemblage zones and radiocarbon-dated horizons at the three sites. 1, Fine silt/clay; 2, coarse silt/sand; 3, gravel; 4, gyttja.

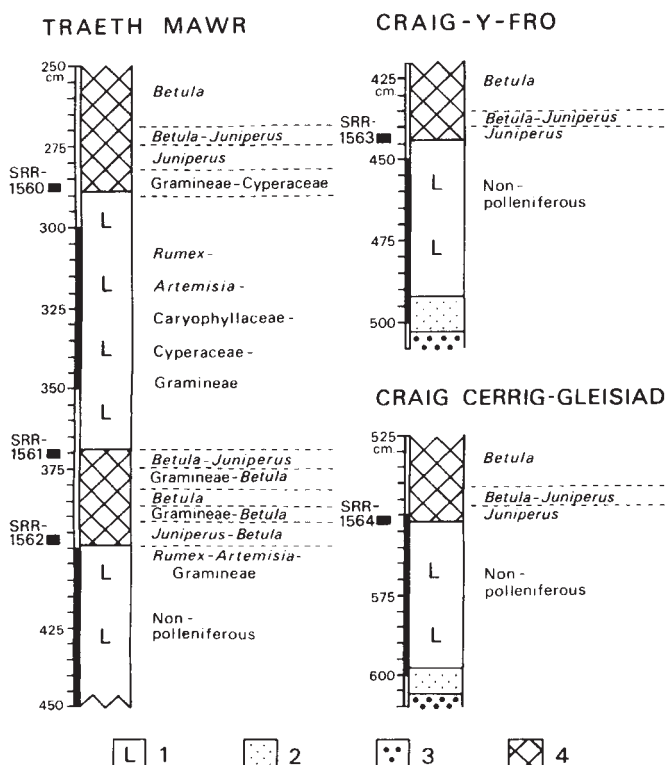


Fig. 2 The Brecon Beacons. Contour interval 100 m. The morainic areas are shown by heavy stipple-shading.

errors on these latter dates mean that the true ages of the dated samples could be somewhat earlier. If correct, however, the radiocarbon determination from the Brecon Beacons and from adjacent regions seem to be slightly younger than the conventional age of 10,300 BP which is generally accepted as marking the close of the Late-Glacial period in Britain, and are also younger than dates which have recently been obtained for the wastage of Loch Lomond Advance ice from Rannoch Moor in western Scotland^{7,26-28}. Collectively, they imply continued minerogenic inwash into lake basins (possibly associated with periglacial activity) for some time after such processes had ceased to operate in other areas of Britain. The reason for this is unclear, for it would be expected that the effects of climatic amelioration, particularly vegetational development and the trend towards soil stability, would have been experienced first in the more southern and western areas of the British Isles. Further research is clearly needed to resolve this paradox.

At the two sites within the moraines, only Flandrian sediments are present. Lithostratigraphically, the sites are very similar (Fig. 3), the absence of Late-Glacial Interstadial sediments clearly implying that both basins contained glacier ice during the Loch Lomond Stadial. The lowermost organic muds at Craig-y-Fro are characterized by high frequencies of Gramineae and *Juniperus* pollen, and by a rising curve for *Betula*. Similar pollen assemblages are found in the basal sediments at Craig Cerrig-gleisiad, except that low values for *Juniperus* are initially recorded in that profile. The lowermost organic deposits at the two sites within the moraines can therefore be correlated biostratigraphically with the early Flandrian sediments at Traeth Mawr.

A radiocarbon date of $10,030 \pm 100$ BP (SRR-1563) was obtained from the basal organic sediments at the deepest point in the Craig-y-Fro basin, a date which agrees well with SRR-1560 at Traeth Mawr. At Craig Cerrig-gleisiad, however, the lowermost gyttja deposits were dated at $10,860 \pm 70$ BP (SRR-1564). This is over 800 yr older than dates obtained from comparable stratigraphic horizons at the other two sites, and is also much older than dates on the Late-Glacial-Flandrian

boundary from elsewhere in Britain. The bedrock around Craig Cerrig-gleisiad is old red sandstone, and there are no known outcrops of calcareous bedrock on the northern side of the Brecon Beacons escarpment. The $\delta^{13}\text{C}$ value for the dated sample (Table 1) lies within the 'normal' range for gyttja material currently observed from European localities²⁹, and thus the possibility of contamination by older carbon would seem to be remote. On the other hand, all rocks are known to contain some elementary carbon which can become incorporated into, and concentrated in, gyttja sediments which are accumulating in areas of newly deglaciated terrain¹⁸. Moreover, it has recently been argued that the $\delta^{13}\text{C}$ value on radiocarbon dates from lake muds can no longer be regarded as a reliable indicator of 'hard water error' in view of the poly-genetic nature of gyttja material^{18,30}. Clearly, therefore, the possibility of a hard water factor in the basal date from Craig Cerrig-gleisiad cannot be excluded, although it is unclear why this should be so when a seemingly 'reliable' date was obtained from a comparable biostratigraphic horizon in the nearby Craig-y-Fro profile (a site located on the same bedrock and with a very similar glacial history).

With the exception of the date from Craig Cerrig-gleisiad, the pollen-stratigraphic and radiocarbon evidence suggests the following sequence of Late-Glacial events. Wastage of the late Devensian ice sheet left the large dead-ice hollow of Traeth Mawr which accumulated organic sediments throughout the mild Late-Glacial Interstadial. Climatic deterioration at $\approx 10,600$ BP led to the break-up of the Interstadial vegetation cover, minerogenic inwash into the Traeth Mawr basin and the recrudescence of small glaciers in the lee of the Brecon Beacons which built moraines beneath Craig-y-Fro and in the cirque of Craig Cerrig-gleisiad. Climatic amelioration at, or shortly before, 10,000 BP prompted the rapid disappearance of these glaciers, the cessation of minerogenic inwash at Traeth Mawr, the onset of organic sediment accumulation in all three basins and the beginnings of Flandrian plant succession.

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Pliocene dispersal of the horse *Equus* and late Cenozoic mammalian dispersal events

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The Cenozoic history of land mammals is marked by brief periods of intercontinental dispersal, termed dispersal events^{1,2}. One of these dispersal events includes the horse *Equus*, that evolved in North America, dispersed to the Old World in the Pliocene, and became a prominent member of Old World Pleistocene communities. *Equus* was derived from a species of the single-toed horse *Pliohippus*, known only from North America³. Timing for the Pliocene dispersal of *Equus* into Europe was questioned⁴ following resolution for the appearance of *Equus* into the Siwalik sequence of Pakistan⁵. It seems that there were at least three major dispersal events of large mammals during the Pliocene (at 1.9, 2.6 and 3.7 Myr). We now report new data that limit the time of *Equus* dispersal and into Europe to the 2.6 Myr dispersal event.

Development of the magnetic polarity time scale^{6,7} and its application in magnetostratigraphy^{8,9} has provided an independent and world-wide time frame for comparison of land mammal dispersal events, and their placement relative to geological events. Figure 1 shows the magnetic polarity time scale for the last five million years, with several North American late Cenozoic biostratigraphic faunal datum events. These biostratigraphic faunal datum events mark the earliest known records for these mammals (such as *Equus*) that dispersed between North America and Eurasia during the late Cenozoic.

Magnetostratigraphic work in Idaho³ and Washington¹⁰ documents the first occurrence in North American deposits of at least five genera of land mammals (*Trigonictis*, *Satherium*, *Parailurus*, *Ursus* and *Bretzia*) whose ancestry and/or an earlier record occurs in Palaeoarctica^{11–15}. These immigrants are first recorded in North America during the interval of 3.5 to 4.0 Myr during the upper part of the Gilbert magnetic chron. The appearance of this diverse group of Palaeoarctic immigrants suggests this interval was a major period of land mammal dispersal between North America and Eurasia.

Early Villafranchian faunas lacking *Equus* and *Elephas* (actually the genus *Mammuthus*) are recognized as chronologically significant¹⁶. Villafranchian land mammal age includes mammal zones MN 16 and 17 (ref. 23) and a pre-E-L-E Villafranchian interval (mammal zone MN 16a) has recently been established¹⁷. Previous divisions of early Villafranchian faunas¹⁸ tended to include faunas with *Equus* and *Mammuthus* for example, Montopoli in Italy and Malusteni in Romania with faunas lacking those genera (for example, Triversa in Italy and Etouaires in France). The Etouaires and overlying Roccaneyra faunas of France are placed in mammal zone MN 16b by Mein¹⁷. *Equus* occurs in the Roccaneyra fauna, but is absent from the Etouaires fauna. The early Villafranchian Viallette fauna in France is comparable to that of Etouaires¹⁹, with both *Equus* and *Mammuthus* absent. The Viallette fauna is overlain by a basalt dated 2.6 Myr (ref. 20) and is underlain by a basalt dated 3.3 Myr. Radiometric results from Etouaires, Roccaneyra and Viallette suggest that the European E-L-E fauna datum (the beginning of mammal zone MN 16b) approximates 2.5 Myr, near the Gauss/Matuyama magnetic chron boundary.

To resolve the correlation of faunas in mammal zone MN 16, and to clarify the European E-L-E faunal datum, we collected

palaeomagnetic samples during the summer of 1979 from stratigraphic sections in Italy yielding the Montopoli and Triversa faunas. Each locality was sampled at five sites, with three samples per site. The samples from the Montopoli section span 32 m, those from the Fornace RDB (Triversa fauna) section span only 13 m. All samples were subjected to both alternating field and thermal magnetization to remove secondary components of magnetization.

The section at Montopoli is very sandy and exposures are poor. Strata suitable for palaeomagnetic sampling were found only after diligent search. Calcareous concretions were sampled at the stratigraphically higher sites. All samples gave natural remanent magnetization (NRM) directions of normal polarity, and a.f. demagnetization did not produce any change in the polarity. Partial thermal demagnetization was attempted on the upper three sites, and pilot samples from the upper two sites changed to reversed polarity very suddenly between 550 and 600 °C. The intensities of these samples were very low, approaching the limits of sensitivity of the magnetometer. Then all remaining samples were thermally demagnetized at 500 °C, at which point the other samples from the upper two sites changed to reversed polarity (although the intensity was very low) with polarity of remaining samples unchanged. The resulting polarity zonation (Fig. 2) is weak because of limitation imposed by the available lithology. However, it is the most reasonable polarity interpretation likely to become available from that section.

The section at the Fornace RDB quarry at Villafranca d'Asti provided finer grained lithologies and stronger magnetic properties but the section available for sampling is shorter. Poorly indurated sandstones occur above and below the sampled section at the Fornace RDB quarry. After a.f. and thermal demagnetization, four of the five sites gave unquestionable reversely magnetized polarity (Fig. 2). The directions from the second highest site gave normal polarity after thermal demagnetization to 500 °C. This site apparently records a normal event within an otherwise reversely magnetized section (Fig. 2).

The Montopoli section is interpreted to represent the upper Gauss and lower Matuyama magnetic chrons, with the boundary occurring about 25 m above the base of the section, 7 m below the top of the section which is the level of the Montopoli fauna. The quarry that yielded the Montopoli fauna was relocated by A. Azzaroli with the help of residents of Montopoli. Our highest palaeomagnetic site is from the level of the quarry, 15 m distant along strike.

The Fornace RDB section is interpreted to represent the middle of the Gauss magnetic chron, with a record of the

Mammoth and Kaena events, separated by a normal magnetozone. The level of mammals recovered from the Fornace RDB quarry approximates the middle of our section. The Arondelli quarry is about 1.5 km distant across the Triversa valley at the same level as the upper part of our section. Azzaroli²¹ correlated the Arondelli and Fornace RDB sections as approximately equivalent. Lithology at the top of our Fornace RDB sampled section is similar to that at the Arondelli quarry. Therefore, the Triversa fauna (based on the Fornace RDB and Arondelli quarry assemblages) is placed in the middle to upper half of our Fornace RDB section, which we correlate with the middle of the Gauss magnetic chron. The polarity zonation of the Fornace RDB section is not definitive for that specific chronological interval; it could represent the Gilbert magnetic chron or the lower Matuyama magnetic chron. Both of those interpretations are rejected, based on faunal similarity of the Triversa fauna with the Etouaires and Viallette faunas of France, and radiometric dates associated with those faunas.

Correlations of the Montopoli and Fornace RDB sections with the magnetic polarity time scale are made with reservation. Interpretations are insecure due to low magnetic intensity of samples from Montopoli, and shortness and non-definitive polarity of the Fornace RDB section. Placement of these sections (and faunas) relative to the magnetic polarity time scale is based as much on biochronological correlation of the faunas as on magnetic polarity zonation.

Magnetic polarity zonation of the Fornace RDB section could have been correlated with the lower Matuyama magnetic chron, which would place the Triversa fauna approximately at the Olduvai event. This correlation is rejected because the Triversa fauna should be earlier than the Montopoli fauna which we correlate with the base of the Matuyama magnetic chron. Faunal similarity of the Montopoli and Triversa faunas is strong, the most significant difference between these faunas is the presence of *Equus* and *Mammuthus* in the Montopoli fauna and their absence from the Triversa fauna. The Triversa fauna is rich and diverse; it includes large mammals like *Tapirus*, *Dicerorhinus*, *Mammut*, and *Anancus*^{21,22}, which are distinct from but comparable in size to *Equus* and *Mammuthus*. If those latter genera had lived in the Po Valley of Italy during the time the sediments that yielded the Triversa fauna were deposited, they would surely be recorded in the fauna.

The Fornace RDB section could also have been correlated with the Gilbert magnetic chron. This correlation is rejected because of an unpublished palaeomagnetic section from the Pichegu quarry about 30 km south of Nîmes, France, that supports a correlation of mammal zone MN 14 (earlier than MN

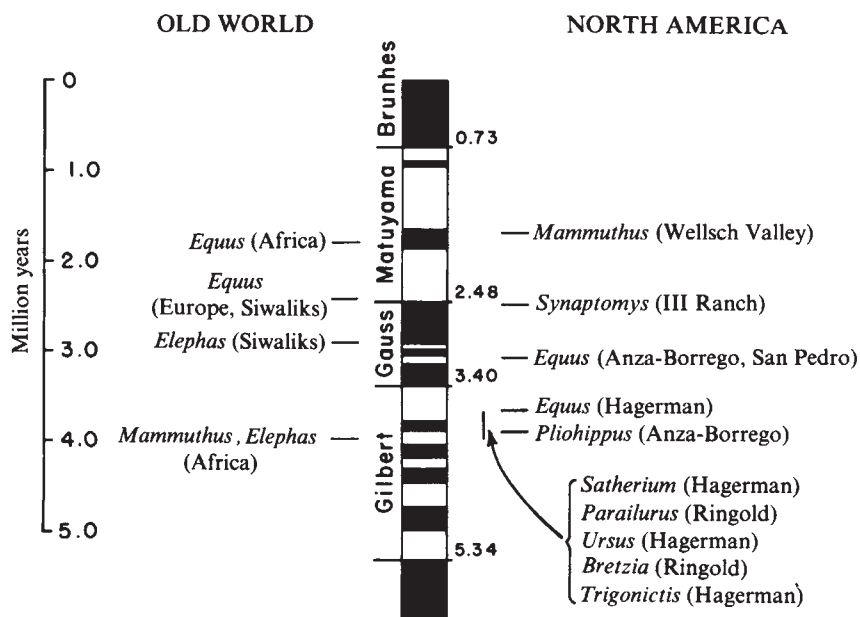


Fig. 1 Important mammalian faunal datum events relative to the dispersal of *Equus*. The central column represents the magnetic polarity time scale (black equals normal polarity and white equals reversed polarity); the Olduvai event is the normal polarity interval at about 1.9 Myr, the Kaena and Mammoth events are the reversed polarity intervals above and below the 3.0 Myr normal polarity event. North American datum events are given for the lowest stratigraphic record of that taxon at the location given in parenthesis. With the exception of *Pliohippus* at Anza-Borrego and *Equus* at Anza-Borrego and San Pedro, the North American faunal data probably represent the earliest North American record for those genera. Old World faunal datum events are believed the earliest known record for those genera on that continent. All faunal datum events are dispersal events with the exception of *Equus* at Hagerman and *Mammuthus* plus *Elephas* in Africa, which are probably evolutionary events. The European E-LE-E (*Equus-Leptobos-Elephas*) faunal datum is placed at the *Equus* faunal datum for Europe and the Siwaliks, at the base of the Matuyama magnetic chron, about 2.5 Myr.

16) with the Gilbert magnetic chron. Suc²⁴ showed that the palynological extinction of plants of the group Taxodiaceae occurs in southern France during the Pliocene, between the small mammal subzones of Hautimagne and Sete. This chronological interval is approximately at the boundary between mammal zones MN 14 and 15. Suc identified the extinction of Taxodiaceae near the top of the Pichegu section (personal communication), which means the main part of that section is correlative, more or less, with mammal zone MN 14. The Pichegu section, which lacks mammals as it is dominantly marine, is reversely magnetized through an interval of more than 50 m stratigraphically below the extinction of Taxodiaceae. We interpret this long magnetozone of reversed polarity, that corresponds broadly with mammal zone MN 14, as part of the Gilbert magnetic chron. Only the Gilbert magnetic chron in the interval of Ruscinian land mammal age (mammal zones MN 14 and 15) has a monotonously uniform reversed polarity zone of that thickness. Therefore, it seems unlikely that the Fornace RDB fauna, in mammal zone MN 16a, is correlative with part of the Gilbert magnetic chron.

These preliminary results, and our interpretations, support a correlation of mammal zone MN 16 with the late Gauss and early Matuyama magnetic chrons, and suggest the division of MN 16a and 16b approximates the Gauss/Matuyama boundary, 2.48 Myr.

Azzaroli²¹ believes that the record of *Equus* and *Mammuthus* from Montopoli is the most primitive and probably earliest record of these mammals in Europe. Thus the appearance of *Equus* and *Mammuthus* in Europe is approximately coincident with their appearance in southern Asia⁵ at about 2.5 to 3.0 Myr. Several early Villafranchian faunas in eastern Europe also have early records of *Equus* and *Mammuthus*, and might be as early as, or earlier than, the Montopoli fauna^{25,26}. Biochronological-palaeomagnetic sequence studies²⁷ of the Ponto-Caspian Basin show the beginning of the *Archidiskodon* Superzone (equivalent to the appearance of *Mammuthus*, and possibly *Equus*) at the Mammoth event (about 3.1 Myr) in that area. Extensive Pliocene-Pleistocene palaeomagnetic studies have been undertaken in southern Tajikistan (near the Russian-Afghanistan border²⁸ where the Navrukho fauna includes the elephant *Mammuthus* (*Archidiskodon*) *gromovi* (also recorded from Montopoli) and *Equus*. The Navrukho fauna is from rocks with reversed magnetic polarity correlated with the lower Matuyama magnetic chron, and is therefore similar in age to the Montopoli fauna.

Our work in Pakistan⁵ demonstrated the appearance of *Equus* and *Mammuthus* in the Oriental zoogeographic region in the late Pliocene (see Fig. 1). We questioned the appearance of these immigrant genera in Europe about one million years before their arrival in southern Asia, but found no definitive data to reject this hypothesis⁴. Results and interpretations given above suggest the coincident appearance of *Equus* and *Mammuthus* in Europe no earlier than about 2.5 Myr.

Equus could not have dispersed before about 3.7 Myr when it evolved from *Pliohippus*; its population density and distribution were probably not sufficient to disperse to Eurasia during the interval 3.7–4.0 Myr, when several Eurasian mammals dispersed to North America. *Equus* became widespread and prominent in the North American faunas after about 3.3 Myr but conditions for dispersal to Asia were apparently not favourable until about 2.6 Myr, shortly before the lemming *Synaptomys* appeared in North America. *Synaptomys* is considered an immigrant to North America and its North American appearance is near-coincident with the south Asian and European appearance of *Equus* (2.5 Myr). Thus, the dispersal history of *Equus* and *Synaptomys* suggest dispersal between North America and Asia about 2.6 Myr.

The discontinuous, sudden, and short duration of these late Cenozoic dispersal events suggests they may be controlled by geological and climatic factors rather than by biological factors. The dispersal history of elephants is similar and supports the concept of more or less widespread and short dispersal events. Elephants evolved in Africa where *Elephas* and *Mammuthus*

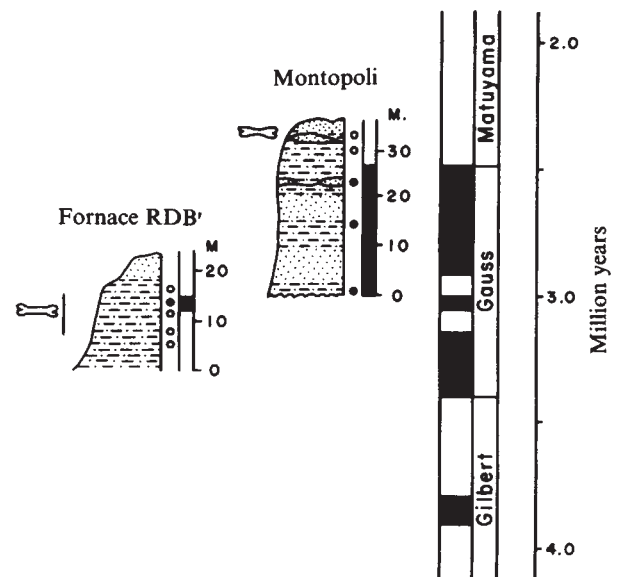


Fig. 2 Magnetic polarity zonation for sections at Montopoli and Fornace RDB quarry (Villafranca d'Asti) in Italy, and correlation of these sections with the magnetic polarity time scale. The Kaena and Mammoth events occur above and below the 3 Myr normal polarity interval. Palaeomagnetic sample sites are shown adjacent to lithologic sections; black circles indicate normal polarity, white circles indicate reversed polarity sites. Location of the Montopoli and Triversa (at Fornace RDB) faunal levels are indicated by bone symbols. The Montopoli fauna is a principal reference for mammal zone MN 16b, with *Equus* present. The Triversa fauna is a principal reference for mammal zone MN 16a, with *Equus* absent.

(*Archidiskodon*) evolved from *Primelephas* at ~4 Myr. *Elephas* dispersed to southern Asia at ~2.9 Myr, slightly before the appearance of *Equus* in southern Asia⁵ and the appearance of *Mammuthus* (*Archidiskodon*) in Europe is approximately coincident with the European appearance of *Equus*. The earliest known record²⁹ of *Mammuthus* in North America is approximately 1.8 Myr. Apparently *Mammuthus* did not enter North America and *Equus* did not enter Africa during the 2.6 Myr dispersal event, because these immigrant populations were too small to warrant further dispersal during that dispersal event. About 1.9 Myr ago, geological and climatic conditions were right for another dispersal event. At that time *Mammuthus* dispersed to North America and *Equus* dispersed to Africa.

The earliest datable occurrences of *Equus* in Africa³⁰ are below the KBS Tuff in the Koobi Fora Formation in Kenya and in member G of the Shungura Formation in Ethiopia. The revised date for the KBS Tuff is 1.81 Myr (ref. 31). The palaeontological evidence (primarily stage of evolution in several lineages of suids) indicates a strong correlation between the KBS Tuff level of the Koobi Fora Formation, member G or H of the Shungura Formation, and Bed 1 of Olduvai Gorge^{32–36}. There is a strong palaeontological and radiometric correlation of these units in the interval of 1.8–2.0 Myr. Thus, the appearance of *Equus* in Africa and the appearance of *Mammuthus* in North America may be coincident. We regard the proximity of these appearances as a significant mammal dispersal event, at about 1.9 Myr.

In summary, it appears the horse *Equus* and the elephant *Mammuthus* evolved at approximately the same time in North America and Africa, respectively, and their dispersal to Eurasia was approximately at the same time. Neither is definitely recorded from Europe or Asia prior to about 3.0 Myr, and both are commonly recorded in fossil deposits of both continents younger than 2.5 Myr. Each genus later dispersed to the continent where the other evolved, but is not recorded on that continent until about 1.9 Myr. Additional data are needed to resolve their appearance in Europe in the interval 2.6–3.0 Myr. The dispersal records and timing of dispersal for these two genera serve as a

model for late Cenozoic intercontinental dispersal of land mammals. They suggest that the history of mammals during that interval is marked by infrequent, widespread, and comparatively short intervals of intercontinental dispersal. Timing for three of these major dispersal events is approximately 1.9, 2.6, and 3.7 Myr. The nature and timing of these major dispersal events suggest they may be triggered by geological or climatic factors.

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Somatic embryogenesis from *Sorghum bicolor* leaves

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Plant regeneration from totipotent cultured cells or protoplasts is a prerequisite for the often proposed genetic modification of plants through somatic cell genetics, and has been achieved in many species. The cereals (and the rest of the Gramineae) have, however, proved to be extremely unresponsive to *in vitro* culture techniques¹. The most convenient source of plant protoplasts is the leaf tissue because it allows the isolation of a large, relatively uniform cell population without the necessity of killing the plant. However, despite considerable efforts, sustained cell division has never been obtained from cereal leaf blade protoplasts². Thus, a crucial question arises of whether leaf cells from

cereals are actually totipotent and consequently, whether they can be useful for somatic cell genetics. We now report that young leaf tissue of a cereal can express totipotency even to the extent of forming somatic embryos. However, the ability to respond is rapidly lost during leaf maturation. We think that this phenomenon can explain many difficulties faced in cereal tissue culture. Moreover, the leaf culture we describe may help to ascertain whether mature cereal cells are merely no longer competent (in the sense of Halperin³) to respond to tested *in vitro* conditions or whether they actually lose their totipotency.

Sorghum bicolor (L.) Moench was chosen for the experiments. (So far, only the variety G522 (Funk Seeds International) found to be responsive in immature embryo and inflorescence culture^{4,5} has been tested.) Plants were grown from seed in test tubes in aseptic conditions for 10 days. Leaves of grasses grow from the base, thus exhibiting a gradient in maturation from base to top. Starting about 0.5 mm above the apical meristem (1.5 mm above insertion of the first foliage leaf), leaves were dissected under a stereo microscope into 1.5-mm long sections. The leaf sections were unrolled and cultured individually on MS

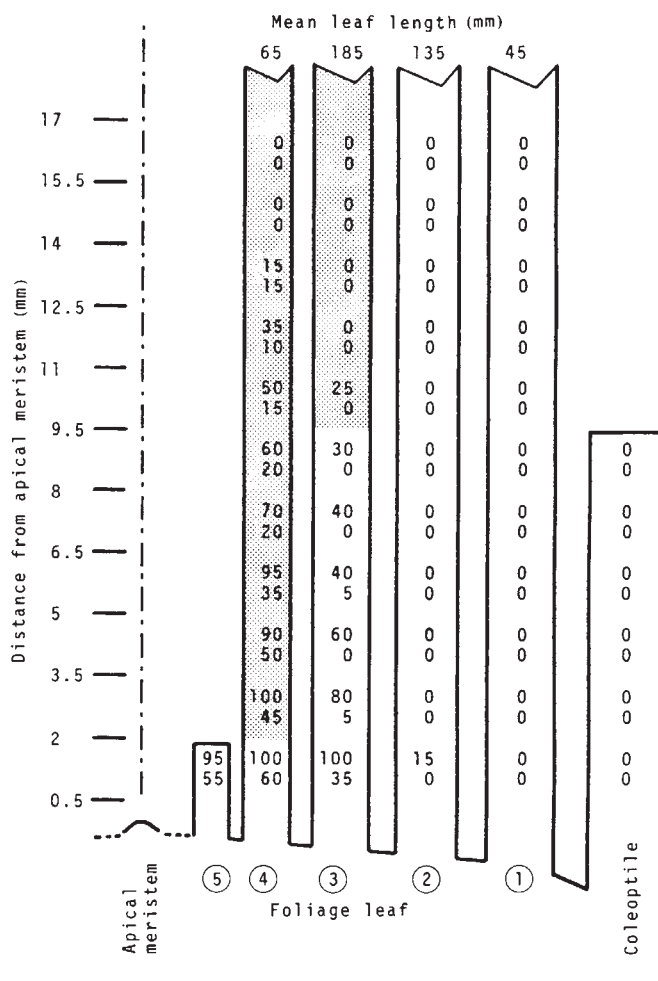
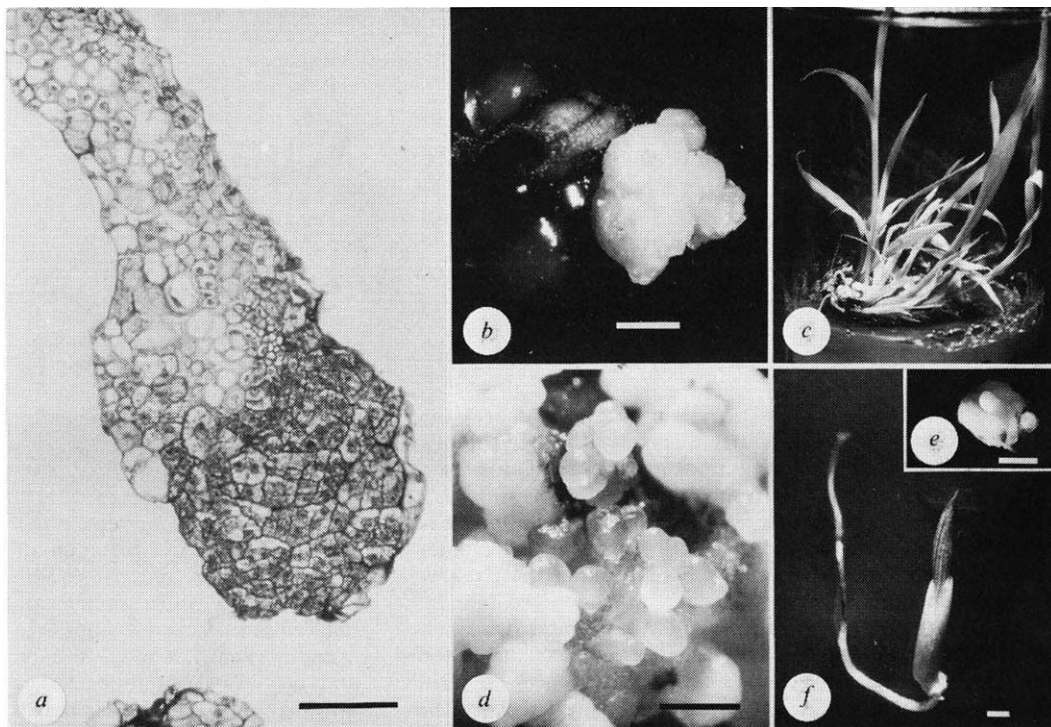


Fig. 1 Diagram of the leaf culture system in *Sorghum bicolor*, showing the response of leaf pieces cultured in the dark at 25 °C. Donor plants were grown from surface sterilized seeds for 10 days on MS medium⁶ with 20 g l⁻¹ sucrose under 16-h days (light provided by fluorescent tubes, 1,500 lx). Twenty equally sized plants were selected. The lower parts of the plants were cut into 1.5-mm long segments and the leaf pieces were placed individually on MS medium supplemented with 2 mg l⁻¹ 2,4-D, 0.1 mg l⁻¹ kinetin and 40 g l⁻¹ sucrose. The leaf blade was clearly distinguishable from the sheath in the first four foliage leaves and is denoted by the shaded area. The experiments were evaluated after 5 weeks culture. In each segment, upper value: % leaf pieces giving rise to callus; lower value: % leaf pieces with macroscopically visible embryogenic tissue.

Fig. 2 *a*, Cross-section through a leaf piece (fourth foliage leaf, ~6 mm above the apical meristem) cultured for 5 days on nutrient medium. Note the cell proliferation underneath a region which has been damaged during excision. Scale bar, 100 μ m. *b*, Callus growing from a leaf piece (fourth foliage leaf, 14 mm above the apical meristem). The non-proliferating tissue produced a dark pigment. *c*, Plant regeneration in the light (800 lx) from white callus tissue after transfer to auxin-free medium. Shoot growth could be accelerated by addition of 0.1 mg l⁻¹ kinetin. *d*, Embryogenic tissue derived from callus shown in *b* after transfer of white compact tissue to a culture medium with reduced 2,4-D concentration. The white globular bodies were able to germinate like zygotic embryos. *e*, Isolated somatic embryo with scutellum, coleoptile and primary root breaking through coleorhiza. Leaf culture origin. *f*, Germinating somatic embryo. In *b*, *d*, *e* and *f*, scale bar, 1 mm.



medium⁶. The youngest leaf that was cultured (fifth foliage leaf) had reached a length of about 2 mm (Fig. 1). In the presence of 2,4-D (1–3 mg l⁻¹) the young leaf pieces proliferated to form a subculturable callus, which consisted for the most part of proliferating yellowish root primordia covered with slimy rootcap-type cells; the roots elongated when 2,4-D was omitted from the medium⁷. In many cases, however, macroscopical examination revealed the presence of a folded, compact, white tissue which, after transfer to auxin-free medium, formed shoots and plantlets (Fig. 2*a–c*). Various additions to the medium such as coconut water, casamino acids, cytokinins, different auxins and gibberellic acid (GA₃), were tested. These modifications gave no significant improvement in growth or were even inferior, although the addition of 0.1 mg l⁻¹ kinetin seemed slightly to increase the relative proportion of the white tissue. After prolonged culture (~5 weeks), especially after transfer to a culture medium with reduced 2,4-D concentration (0.5 mg l⁻¹), the white tissue formed globular structures (Fig. 2*d*). Many of these structures resembled zygotic embryos in having a scutellum and a clearly defined root-shoot axis. After transfer to a medium without auxin, optionally supplemented with 0.1 mg l⁻¹ kinetin, the structures were able to germinate like zygotic embryos (Fig. 2*e, f*). In this respect, the leaf cultures did not differ from cultures derived from the scutellum of immature embryos⁴.

The response of different leaf pieces is summarized in Fig. 1. It can be seen that the further the 'distance' (spatial and temporal) from the apical meristem, the less responsive the tissue became. This pattern of response correlated with the pattern of leaf blade and leaf sheath maturation described by plant morphologists^{8,9}.

Cytological examination of cultured immature leaf pieces derived from the fourth foliage leaf revealed that developing mesophyll, bundle sheath and occasionally epidermal cells close to lesions caused by excision were prone to proliferate (Fig. 2*a*). In explants taken from the third foliage leaf, proliferation was more restricted to cells closely associated with the vascular bundles, and resulted in less white, compact tissue (Fig. 1). It seems, therefore, that the embryogenic tissue is formed by mesophyll and/or epidermal cells.

The calluses which have been obtained from immature leaf pieces consist of a mass of proliferating primordia (shoot-

embryo and/or root primordia). Full expression of morphogenesis is inhibited by the hormone (2,4-D in this case) essential for stimulation of callus growth, but the primordia can be released by reduction and omission of the hormone. The occurrence of similar responses in morphogenetic cultures of dicots has also been recently discussed¹⁰. Our findings suggest that the grasses differ from dicots in that the cells have to come from a very immature tissue. In our experiments, totipotent cells were clearly part of the growing leaf blade as well as the leaf sheath, but only in the two least mature leaves. (Root-type callus cultures have been induced from basal (immature) parts of rice leaf sheaths¹¹.) The recent finding of Saalbach and Koblitz¹², who in a single experiment obtained shoots from a cultured barley leaf, could be explained by proliferation of cells from a leaf that happened to come from close to the apical meristem.

Competence *in vitro* may be correlated with continuing meristematic activity *in vivo*. In fact, in grasses, cell proliferation *in vitro* has only been observed in tissues that are close to an undifferentiated state¹³. Thus, the process which in research on dicots has been termed 'dedifferentiation' has most critical implications for cereal tissue culture. The use of the leaf culture system may enable us to define more precisely the step in differentiation leading to loss of competence (if not totipotency) and thereby possibly overcome the barrier which prevents the mature cells from responding *in vitro*.

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Circadian rhythmicity participates in the photoperiodic determination of diapause in spider mites

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The role of circadian rhythmicity in photoperiodic processes in plants and animals is still poorly understood. Involvement of circadian rhythmicity in photoperiodism has been demonstrated for a number of plants^{1,2}, two mammals^{3,4}, a number of birds^{5,6}, and some species of insects⁷⁻¹¹. Three theoretical alternatives have been suggested with regard to the form this involvement may take, with emphasis on the possibility that the circadian system is concerned with photoperiodic time measurement¹²⁻¹⁴. Most of the evidence for the involvement of the circadian system in both plant and animal photoperiodism comes from experiments with nondiel photoperiods—light/dark cycles with period lengths other than 24 h. The experimental design which has been used most frequently is the so-called resonance technique, in which the light component of a light/dark cycle is held constant and the dark component is varied over a wide range in successive experiments, to provide cycles with period lengths up to 72 h or more. Rhythmic variations in the photoperiodic response with peaks about 24 h apart are evidence for the involvement of circadian rhythmicity in the photoperiodic process studied. However, in some plants¹, a reptile¹⁵, and a number of insect species¹⁶⁻²⁰, the resonance technique failed to demonstrate any influence of circadian rhythmicity on photoperiodism. Using the same technique we have now found a rhythmic response in the incidence of diapause in the common spider mite, *Tetranychus urticae*, thus showing that in mites also, circadian rhythmicity is involved in the photoperiodic reaction.

Diapause in *T. urticae* is of the facultative type and occurs normally in adult females which have experienced short-day photoperiods during their larval and nymphal stages of development²¹. The most conspicuous characteristic of the diapause state in *T. urticae* is the deeply orange colour of the diapausing females, as compared with the greenish-yellow of the 'summer' females. Other features of the diapause are arrest of oviposition, cessation of feeding, voiding of the intestinal tract and changes in behaviour. For the experiments, the mites were kept on detached leaf cultures of beans (*Phaseolus vulgaris*). Resonance experiments were performed at 19°C; photoperiodic cycles consisted of a fixed light phase of 8 h duration and dark phases which were varied over 4–84 h, the duration of darkness being increased in steps of 4 h in the repetitions of the experiment. Approximately 250 mites were used in each photoperiodic treatment and each photoperiodic regime was sustained throughout the complete development of the mites.

The results presented in Fig. 1 clearly show that, with a fixed duration of the light phase, the incidence of diapause in *T. urticae* is a rhythmic function of the duration of darkness. Peaks of high diapause incidence recur with cycle lengths (duration of light plus dark) of about 24, 44, 64 and 84 h. Diapause induction is completely averted in photoperiodic cycles consisting of 8 h light combined with dark phases of 4–9, 24–28, 44–48 and 60–64 h. Apart from the cycles with night lengths of 4–9 h, in all photoperiodic regimes the duration of darkness exceeded the critical nightlength, which is 10 h in the strain of mites used. The conclusion from these experiments is that in *T. urticae* circadian rhythmicity is somehow involved in the photoperiodic determination of diapause. Thus we demonstrate here, for the first

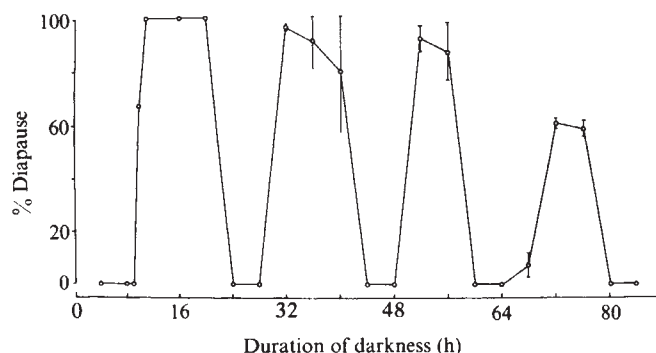


Fig. 1 Diapause incidence in *T. urticae* in cycles of 8 h of light and (abscissa) different durations of darkness. Vertical lines represent ± 1 s.e.m.

time, a functional connection between the circadian system and photoperiodism in a representative of the Acarina or mites.

The results presented here are remarkable in several respects. A sequence of four resonance peaks as shown in Fig. 1 has not been observed in an animal before. The third and fourth peak are only slightly reduced in height in comparison with the first and second. In continuous darkness diapause incidence in these mites is 0% (ref. 21). This means that photoperiodic induction still takes place in cycle lengths of over 80 h. Of these long cycles at 19°C, only three or four are perceived by the mites during the photoperiodic sensitive larval and nymphal stages. However, previous experiments with *T. urticae* have shown that the summation of photoperiodic information may already be saturated with three photoperiodic cycles²¹, which might explain the presence of a fourth resonance peak in the experiments presented here.

Another divergence from the results obtained with other organisms is the fact that the resonance peaks in the experiment with *T. urticae* as shown in Fig. 1 are only about 20 h apart. Resonance experiments with plants, mammals, birds, and insects have shown peak intervals of about 24 h (refs 1–6, 22, 23). If we assume that the peaks of diapause incidence in Fig. 1 result from an influence of the circadian system on the photoperiodic reaction in these mites, the circadian rhythm involved may have a free-running period of only about 20 h. This value is exceptionally low when compared with the values of the periods of all known kinds of free-running circadian rhythms, which tend to be closer to 24 h and normally vary over ~22–27 h (refs 22, 23).

Earlier experiments with *T. urticae*, such as experiments with temperature cycles in continuous darkness²¹, with light interruptions in various long nights, and with two light pulses of equal duration in each photoperiodic cycle, could be explained on the assumption that circadian rhythmicity is not involved in photoperiodic time measurement in these mites^{24,25}. In work on the fruit tree red spider mite, *Panonychus ulmi*, Lees^{26,27} found no indication for the participation of circadian rhythms in the photoperiodic reaction. The divergent results with the two spider mite species are difficult to explain in terms of a mechanism of photoperiodic time measurement. A full account of the results presented here, with considerations on photoperiodic time measurement in spider mites, must await further experiments with nondiel photoperiods in *T. urticae*, which are now under way, and will be published elsewhere.

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A test of evolutionary theories of senescence

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Senescence is the post-maturation decline in survivorship and fecundity that accompanies advancing age. Two main evolutionary theories have been proposed to account for senescence. (1) The mutation-accumulation theory. Deleterious mutations exerting their effects only late in life would tend to accumulate, because of their minimal effects on fitness^{1,2}. More precisely, exclusively late-acting deleterious mutations will attain higher equilibrium frequencies under mutation-selection balance than will mutations that act early, resulting in lower mean values for fitness components late in life (ref. 3, p. 218). Medawar¹ emphasized the possibility that this effect would be enhanced by selection of modifiers that postpone the age of onset of genetic diseases. (2) The pleiotropy theory. Williams⁴ suggested that many of the genes with beneficial effects on early fitness components have pleiotropic deleterious effects on late fitness components, but are nevertheless favoured by natural selection. (These theories are based on the decline with age in the effect of age-specific fitness-component changes on total fitness³ (ref. 3, pp. 206–214 and refs 4, 5). Either or both of these theories could apply in any particular population.) Selection experiments in *Drosophila*⁶ and *Tribolium*⁷ support the pleiotropy theory, although one such experiment gave results that only bordered on significance⁸, but the mutation-accumulation theory has never been tested. The present results provide evidence for the pleiotropy theory, but do not support the mutation-accumulation theory.

Tests of evolutionary theories with randomly breeding laboratory populations, as in the present case, require that they be maintained without significant inbreeding after sampling from a long-standing natural population. In August 1975, 400 *Drosophila melanogaster* adults were sampled from the Amherst, Massachusetts, population studies by Ives⁹. There is good evidence that this population has been continuously endemic in the Amherst area since about 1930 (ref. 9). The flies were maintained in the laboratory at 25 °C in 10–16 one-third-pint milk bottles containing Lewis medium and powdered yeast.

Discrete generations were used, with 13–15 days from oviposition to adult egg laying. Population size bottlenecks did not occur.

Female phenotypes were assayed as follows. Egg laying counts (24 h) and longevity were recorded from the fourth day of adult life. One male and one female were kept in plastic tubes, under constant illumination at 25 °C, with high-agar charcoal-coloured medium. The surface of the medium was prepared with a yeast paste and a small amount of acetic acid. Daily transfers were performed using CO₂ anaesthesia.

The additive genetic variance (V_A) of a fitness component such as daily egg lay should increase with age if the mutation-accumulation theory is correct. (This is because the additive genetic variance contributed by a di-allelic locus with one allele close to fixation increases with the frequency of the rare allele (ref. 10, p. 135 and ref. 11). The equilibrium frequency of a deleterious allele maintained in the population by recurrent mutation will be higher the later its age of action, if its expression is limited to certain ages^{1–3}.) Unfortunately, there is a difficult problem concerning the scale on which daily egg lay should be measured so as to test the hypothesis of an increase in V_A with age. Mean daily egg lay shows a senescent decline, from around 70 eggs per day for the first day of assay to about 30 eggs per day

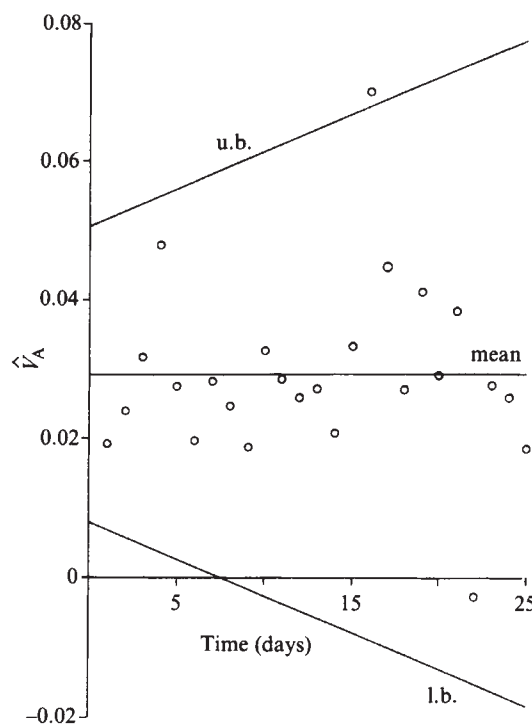


Fig. 1 Estimates of additive genetic variance ($\hat{V}_A$) for egg laying over 24 h. Only data from females which survived the 24 h and laid at least one egg during this period were used, since approximate normality of the data is required for analysis of variance. The data were transformed using the function $x' = \ln(x + 23.81)$, so as to remove the scale effects arising from the fall in egg laying during the 25-day period of assay¹². The estimates were obtained using a sibling analysis of variance with unbalanced cell allocation (ref. 13, p. 459). The total number of individuals scored falls from 1,099 on day 1 to 396 on day 25. The overall mean for $\hat{V}_A$ during the assay period is 0.02925, which is plotted. The least squares linear regression slope for $\hat{V}_A$ on age has a slope of -0.00002 ± 0.00072 , with 95% confidence, which has a t value of -0.0599 , with 23 degrees of freedom. This is not significant at any reasonable level of probability. The best estimate for V_A over the period is its mean value. Because of a confounding design variable, confidence intervals for this estimate had to be estimated on an *ad hoc* basis using the normal equations for the unbalanced design¹⁴, giving the upper and lower bounds (u.b. and l.b.) for daily estimates of V_A shown.

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for the last day of assay shown in Fig. 1. V_A for untransformed daily egg lay shows a parallel decline. This presumably reflects the fact that a gene with a given effect on fecundity, expressed as a proportion of the population mean, will have a higher absolute effect when the mean is high than when it is low. This suggests the use of a logarithmic transformation of the data. We chose to use the transform $x' = \ln(x + 23.81)$, which removes the relationship between mean and gross phenotypic variance in daily egg lay¹².

Figure 1 shows estimates of V_A on the transformed scale, plotted as a function of age. The upper 95% confidence limit for the regression slope of V_A on age is only 0.0070, whereas the mean of V_A is about 0.0292. There is thus little scope for a substantial increase in V_A with age on the basis of these data. Although the method of transformation used here is a standard procedure in quantitative genetics¹², it is open to the objection that it may disguise a relationship between V_A and age on some other, 'true' scale of measurement. A detailed model of the mutation-accumulation theory, to be presented elsewhere, shows that a roughly threefold rise in V_A on our scale, between first and last day of assay, would be needed to account for the observed decline in mean fecundity. This assumes that the effects of mutations (expressed as proportions of mean fecundity) are similar at different ages. Our results therefore imply either that the mutation-accumulation theory is false, or that the late-acting mutations have much smaller proportional effects on fecundity than early-acting ones.

A test of the alternative theory of pleiotropic gene effects⁴ was based on the principle⁶⁻⁸ that selection for increased reproductive output at later ages should result in decreased fertility at early ages. Figure 2 and Table 1 give the results of an experiment which was contrived so that the effects of selection for late reproduction could be detected at any age. These results show that increases in later reproductive output are associated with decreases in early reproductive output, lowered overall reproductive rate, and increased longevity. This concurs with the predictions of the pleiotropy theory of senescence. Comparable results have been obtained in other experiments on *Drosophila* (see refs 7, 15 and Fig. 7 of ref. 16), although the latter authors favoured an interpretation in terms of maternally transmitted, non-mendelian 'ageing factors'. The increase in late fecundity of flies bred from older parents observed in all three experiments, and the increased longevity of such flies that we have detected, are difficult to reconcile with such an interpretation. The only experiment designed as a critical test for the existence of non-mendelian ageing factors in *Drosophila* (using an inbred line to eliminate genetic variability) yielded negative results¹⁷. Our data provide two lines of evidence in favour of a conventional mendelian interpretation. First, the analysis of variance of the sibling experiment showed similar values for the between-dam and between-sire components of variance (ref. 10, p. 173) in daily fecundity, so that there was no evidence for maternal inheritance¹⁸. Second, analysis of the additive genetic correlations between fecundity at different ages, and between fecundity at various ages and longevity, gave several high negative values, broadly consistent with the results of the selection experiments¹⁸. Thus, there seems little reason to think that our

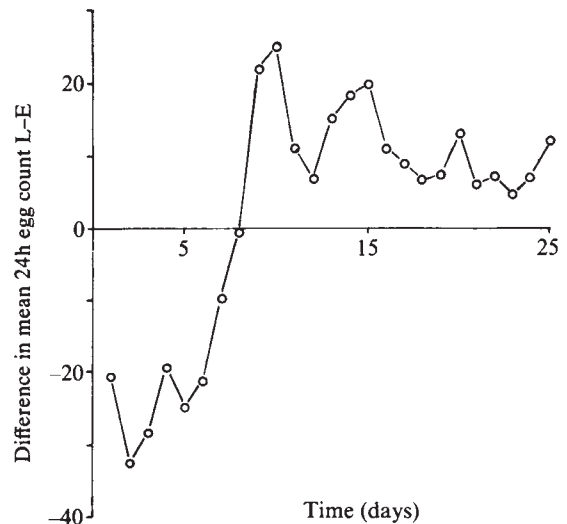


Fig. 2 The ordinate is the difference in mean 24 h egg counts between a sample from a population reproduced at late ages, L, and a sample from a population reproduced at early ages, E. The E population was the base population, maintained as described in the text. The L population was obtained from eggs laid by one generation of the E population. It was then maintained for 12 generations in the same conditions as the E population, but using eggs laid by adults surviving at least 21 days from pupal emergence. Samples of about 100 females were then obtained from each population, and assayed as described in the text. Many of the differences in daily egg-laying shown are statistically significant. Table 1 gives other significant differences between the samples.

selection responses were due to causes other than selection acting on pre-existing, mendelian variability.

We conclude, therefore, that there is genetic variability in female life-history components in *Drosophila*, of the sort postulated by Williams' pleiotropy theory of senescence⁴. This does not, of course, necessarily mean that the established pattern of senescence in *Drosophila* females has been evolved as the result of selection acting on such genes, but this seems likely unless the present properties of genetic variation in *Drosophila* differ radically from those in the past. It therefore seems reasonable to suggest that senescence in *Drosophila* is due to the late-acting deleterious effects of genes which are favoured by natural selection because of beneficial effects at early ages. It does not necessarily follow from this that senescence in all species is due to such pleiotropy. However, that hypothesis is definitely worth testing, in view of the results given here. Articles presenting these experiments in greater detail will appear elsewhere¹⁸.

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Table 1 Significant differences between samples from the E and L populations of Fig. 2*

Character	L mean	E mean	d.f.	Results of t-test
Five-day total egg laying:				
Assay days 1-5	421.91	551.30	201	-12.364
Assay days 11-15	392.19	323.37	190	4.044
Assay days 16-20	286.69	238.78	165	2.431
Assay days 21-25	182.85	136.79	110	2.515
Longevity in assays days	30.25	26.79	200	2.283
Rate of laying during assay	68.61	76.93	200	-3.327

* $P < 0.05$.

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Chimaeras of *Drosophila melanogaster* obtained by injection of haploid nuclei

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Since the observation by Bridges^{1,2} of a few mosaics containing haploid tissue in *Drosophila*, specimens supposedly haploid have rarely been found³⁻⁵. A technique for the easy production of haploid animals or tissues could have important applications. We have now successfully designed such a technique by the production of chimaeras obtained by transplantation of haploid nuclei from a maternal effect mutant that produces haploid embryos. We have used this technique to test the proposal of Jack and Judd⁶ that the *zeste* locus of *Drosophila melanogaster* cannot repress the activity of unpaired alleles at the *white* locus.

The mutation 1182 described by M.G. *et al.*⁷ and by Zalokar *et al.*⁸ produces by a maternal effect haploid embryos that never develop into complete larvae. Recently⁹, Debec obtained permanent haploid cell cultures from these embryos and also recovered a mutant stock that is fully penetrant at any temperature. We have again checked the characteristics of eggs of this new strain renamed here *maternal haploid* (*mh*). The technique of fixation and staining is as described by Zalokar *et al.*¹⁰. In 25 young eggs examined (stages 1-4 of Zalokar and Erk¹¹), the 3 polar nuclei were found in their normal place in the anterior part of the cortex. Thirteen other eggs were suitable for a karyotypic analysis (stages 9-13) and all showed a set of haploid chromosomes with an X chromosome. From 200 untreated eggs, 183 developed to blastoderm; gastrulation was abnormal and only 22 differentiated further to form embryos with some muscular movements and abnormal segments.

To obtain the chimaeras, two to six nuclei from eggs at stages 10-14 of Zalokar and Erk¹¹ laid by homozygous *mh* females crossed to *Ki e*¹¹ males, were injected into *y f*^{36a}; *mwh jv* host eggs at stages 2-8. We used the transplantation technique of Zalokar¹² as described in Santamaría¹³. From 1,046 injected eggs, 429 adults developed, of which 44 had patches of tissue phenotypically *y*⁺ *f*⁺; *mwh*⁺ *jv*⁺ *Ki*⁺ *e*⁺. Twelve more chimaeric larvae died before imaginal differentiation, and were recognized by black mouth parts and bigger, black intersegmental hooks. The fact that all these chimaeras were neither *kinked* nor *ebony* implies that the initiation of mitosis can be triggered by the entrance of the spermatozoon (virgin eggs from *mh* mothers never develop) and that the haploid nuclei can support development at least until the gastrula stage. The phenomenon differs from that in known parthenogenetic *Drosophila* species¹⁴⁻¹⁷ in which a female pronucleus becomes diploid or the products of meiosis fuse and afterwards undergo normal development.

In 24 chimaeras out of the 44, neither cells nor organs of wild-type phenotype were obviously smaller than those of homozygous *mh* females, indicating that the injected nuclei probably diploidize. In fact, in the permanent cell cultures produced by Debec⁹ from haploid embryos, some cells diploidize. Moreover, in the Subtenly experiments¹⁸ involving transplantation of nuclei from haploid blastulae into enucleated eggs of *Rana pipiens*, some of them develop into typical haploids and others into homozygous diploids.

Of the 44 chimaeras, 17 showed reduced cell size in all of the donor tissue. In four the organ was smaller than in controls, and in eight the *mh* organ carried more bristles than controls. Some examples are given in Table 1 and Fig. 1. These three modifications were generally found in the same mosaics, but in small patches the size of the organ or the number of pattern elements could not always be distinguished from those of controls.

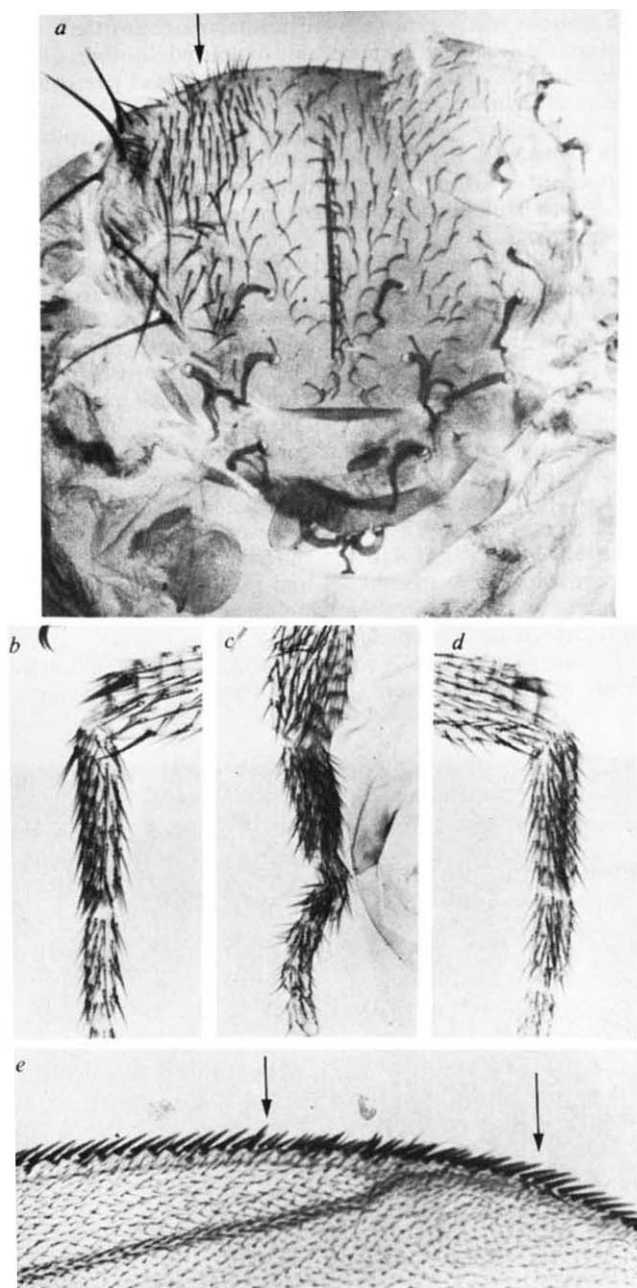


Fig. 1 *a*, Patch of haploid tissue, marked with arrows, in the notum, showing increase in the number of bristles. The host was *y, f*^{36a}; *mwh jv*. *b*, Normal first leg basitarsus of a *mh* female. *c, d*, The first legs of haploid tissue. The basitarsus shows shortening and an increase in the number of bristles. Both were female. *e*, Chimaeric medial triple row formed by reduced, marked with arrows, and normal bristles, both *mh*.

In three more chimaeras in which the size of most of the *mh* cells was normal, a clone also of *mh* cells differentiated in which the bristles were clearly smaller (Fig. 1*e*).

The reduced size of haploid cells and the reduction of organ size are generally found in plants and animals¹⁹, including *Drosophila*^{1,2}. However, we believe the variation in the number of elements in the pattern to be new. In *Drosophila*, Held²⁰ observed a reduction in the number of pattern elements in triploid flies. It is therefore likely that haploid organs would produce more bristles. As we found chimaeras with the expected characteristics of haploid tissue, it seems reasonable to assume that we are really dealing with haploid differentiation. Moreover, we can argue that the observation of chimaeras with

mh tissue in which some cells are normal in size and others small, indicates that some of them became diploid while others did not, therefore excluding the possibility that the small size could be due to other mutations arisen in the strain.

All cuticular regions can be formed by supposedly haploid *mh* cells. However, the fact that complete haploid embryos die implies either that not all embryonic processes can be supported by the *mh* haploid nuclei, or that a maternal effect prevents this development.

Eight chimaeras were formed by *mh* cells in the region of the sex comb. All the chimaeras in the first leg basitarsus were female, but only two had indications of haploidy. Three more chimaeras had a seventh tergite, which is a female characteristic. Our five *mh* chimaeric genitalia, two were clearly haploid and female, one probably diploid and female, one half male and half female and another one completely male.

Three possible explanations for the maleness are: (1) the formation of X/O; 2A cells, (2) the formation of intersexes 2X; 3A, (3) an ambiguity in the possibilities of differentiation of haploid cells. The well established law of sex determination as a function of the ratio of X to A²¹, the fact that X/O males appear in parthenogenetic species¹⁴⁻¹⁷ and the lack of indications of haploidy or triploidy in these chimaeras indicate that the first hypothesis is the most plausible.

If monoploidy of an X is possible, this suggests that monoploidy of a 4th chromosome is also possible in otherwise diploid

Table 1 Comparison between some parameters obtained from 10 homozygous *mh* females and some individual chimaeras

	Control	Chimaeras
Bristles in FLB	105.2 ± 7.7	175, 156
Wing hairs	18.5 ± 2.2	26.4 ± 5.8
Length bristle AP	105.3 ± 4.2	71
Acrosticals	134.3 ± 4.8	156, 195
Bristles in the MTR	93 ± 1.5	77, 73

FLB: first leg basitarsus. Hairs per wing are counted in a square placed in the ocular and between veins L II and L III. Length of the biggest bristle of the anal plate (AP) was measured with an ocular grid. Acrosticals were counted on photographs of the flies. The number of bristles in the medial triple row (MTR) is always reduced in reduced organs probably due to the continuity of elements and as a consequence of the shortening of the wing. In the rest of the organs in which the bristles are interspersed between hairs the phenomenon is the reverse, as in the FLB.

cells. Haplo-4 tissue would be *Minute* and produce small organs which might be difficult to distinguish from haploid regions. However, haplo-4 individuals never show an increase of pattern elements; also, *Minute* tissue should either disappear by cell competition²² or fill an entire compartment when all the cells of the original anlage are of donor origin²³; this is not the case in most of our haploid chimaeras. Thus, the results presented here indicate that haploid cells can probably develop and differentiate in all the adult cuticular tissue.

We hope this technique will prove useful in the analysis of such genetic phenomena as transvection effect, variegation and cis regulation. We have used it to provide new evidence in favour of the thesis of Jack and Judd⁶: in a *zeste* individual, paired *white*⁺ alleles fail to be expressed while unpaired *white*⁺ alleles are expressed normally. The repression of *white*⁺ alleles by *zeste* appears (according to Jack and Judd⁶) to be similar to the transvection described by Lewis²⁴. According to this hypothesis, one would predict that haploid *zeste* cells must be *zeste*⁺ in phenotype. Twenty-two chimaeras were obtained from injection of nuclei from eggs laid by *mh z* females crossed to *Kie*¹¹ males into *y w sn* eggs. Eleven of these showed a *y*⁺ *w*⁺ *sn*⁺; *Ki*⁺ *e*⁺ patch in the eye region: six were *zeste*, four were *zeste*⁺ and one was *zeste* and *zeste*⁺. All of the *z*⁺ mosaic areas had reduced cell and organ size (Fig. 2), whereas the *z* areas were normal; therefore, the possibility that the *zeste*⁺ phenotype is due to the lack of an X chromosome seems unlikely. The fact that the tissue with normal cell size was *zeste* and that tissue with small cells was *zeste*⁺ provides further support in favour of the haploid nature of the *zeste*⁺ patches.

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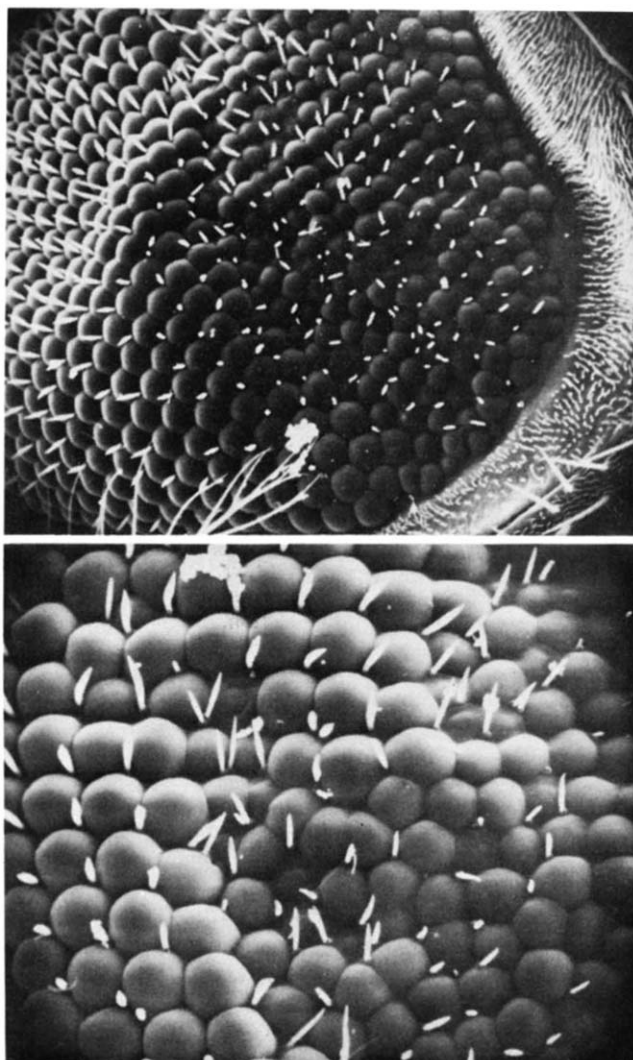


Fig. 2 Scanning electron microscope photograph of *z mh* haploid tissue, *z*⁺ in phenotype, showing reduction of facet size and disturbed bristle pattern.

Gonadotropin releasing hormone agonists stimulate meiotic maturation of follicle-enclosed rat oocytes *in vitro*

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Although the principal function of gonadotropin releasing hormone (GnRH) is to stimulate the pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH), there is evidence that agonistic analogues of GnRH directly inhibit steroidogenesis in the testis¹ and ovary²⁻⁵. On the other hand, Clark *et al.*⁶ have demonstrated that GnRH and two agonists have a marked stimulatory effect on prostaglandin synthesis by granulosa cells isolated from immature rats treated with pregnant mare's serum gonadotropin (PMSG). Stimulation by these compounds was distinct from that by LH in that no changes in cyclic AMP production were detected. Thus it seems important to investigate the effect of these peptides on other aspects of ovarian function, for example oocyte maturation. Mammalian oocytes are arrested in the dictyate stage of the first meiotic prophase, and meiosis (oocyte maturation) normally resumes in preovulatory follicles as a consequence of the surge of LH and FSH. This maturation can also be initiated *in vitro* by the addition of gonadotropins to isolated preovulatory follicles^{7,8}, and is accompanied by an increase in the production of lactate⁸. We now report that GnRH and two potent agonists⁹ stimulate meiosis *in vitro* in follicle-enclosed oocytes in a dose-dependent and specific manner, and also cause an increase of lactate accumulation during incubation.

Immature, 26-day-old female rats were injected subcutaneously with 10 IU PMSG (Sigma). After 48 h we isolated the intact preovulatory follicles (8 to 14 from each rat) by microdissection. We incubated them (4 to 7 follicles per flask) in 0.7 ml modified Krebs bicarbonate buffer, with and without test substances, at 37 °C for 2–10 h as described earlier⁸. Following incubation, the medium was frozen for later analysis of lactate content¹⁰, and the oocytes were recovered to determine their stage of meiosis. We examined the living oocytes by Nomarski interference contrast microscopy and in certain cases fixed and stained them according to Donahue¹¹. Oocytes were put into one of three categories: (1) immature, with germinal vesicle (GV); (2) maturing, with GV breakdown (GVB); (3) mature, with first polar body (PB). The following test substances were added, as indicated, directly to the incubation media: ovine LH (NIH-LH-S18); synthetic GnRH (LHRH batch 750125, Ferring); [D-Ala⁶, des-Gly-NH₂]¹GnRH ethylamide (analogue I, Beckman); [D-Leu⁶, des-Gly-NH₂]¹GnRH ethylamide (analogue II, Abbott); TRH Roche (Hoffman-LaRoche); oxytocin (Syntocinon, Sandoz) and somatostatin (batch RAA 14511, Kabi).

In control media, the follicle-enclosed oocytes remained immature, whereas LH, used as a positive control, induced maturation with 99% GVB after 4 h incubation (Table 1). Preliminary experiments failed to show any influence of analogue I on the LH response. Somewhat unexpectedly, GnRH as well as the GnRH agonists added alone were potent stimulators of meiosis (Fig. 1). With GVB as an endpoint, the ED₅₀ was 492 ng ml⁻¹, 0.588 ng ml⁻¹ and 0.898 ng ml⁻¹ for GnRH, analogue I

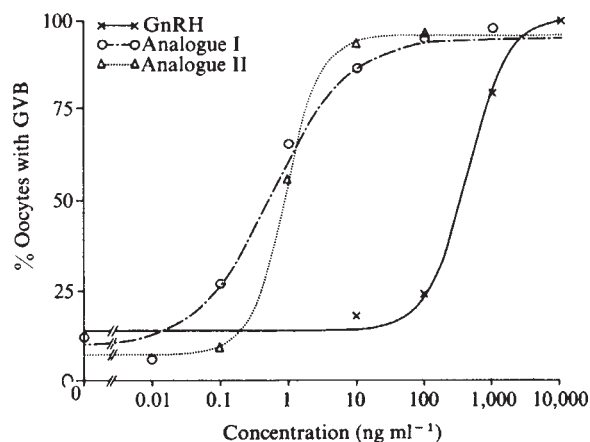


Fig. 1 Dose-response curve of GnRH, analogues I and II on germinal vesicle breakdown (GVB) of follicle-enclosed oocytes incubated for 4 h. The percentage of oocytes with GVB of the total number examined was determined as described in the text. The total number of oocytes analysed were 82, 142, 261 and 141 for control, GnRH, analogues I and II, respectively. Dose-response curves were fitted to the data by computer program using a nonlinear least-squares regression²⁰.

and analogue II, respectively. The time course of maturation induced by analogue I was similar to that induced by LH (Table 1). Analogue I caused complete maturation during 10 h incubation as evidenced by PB formation (Table 1). Synthetic TRH, oxytocin and somatostatin did not stimulate meiosis, indicating the specificity of the effect of GnRH and the agonists (Table 2).

Although not studied quantitatively, it was clear that GnRH and both analogues also produced dispersal and mucification of the cumulus oophorus, as was previously described for LH and FSH¹².

We also found that lactate accumulation was markedly stimulated by LH (Fig. 2) as reported earlier^{8,10}. GnRH, analogue I and II, but not TRH, also enhanced lactate accumulation. The maximal response, however, was considerably less than the maximal response produced by LH. When we tested LH together with analogue I, we did not find any significant additivity.

These data clearly show that GnRH has a stimulatory effect on meiotic maturation of follicle-enclosed rat oocytes. When we initiated these experiments we did not know what results to expect, as GnRH and agonistic analogues have been shown to have both direct inhibitory²⁻⁵ and direct stimulatory⁶ effects on different parameters of ovarian function. In our first experiments we found that these substances did not inhibit spontaneous maturation of isolated rat oocytes, nor did they inhibit

Table 1 Time course of meiotic maturation induced by analogue I and LH

	Per cent with GVB 2 h	4 h	Per cent with PB 10 h
Control	8 (50)	16 (110)	12 (43)
Analogue I	22 (50)	98 (110)	75 (44)
LH	26 (38)*	99 (78)	63 (75)*

Follicle-enclosed oocytes were incubated for the times indicated in absence or presence of analogue I (1 µg ml⁻¹) or LH (1 µg ml⁻¹). Percentage of oocytes with germinal vesicle breakdown (GVB) or first polar body (PB) of the total number examined (in parentheses) was determined as described in the text.

* Data from ref 8.

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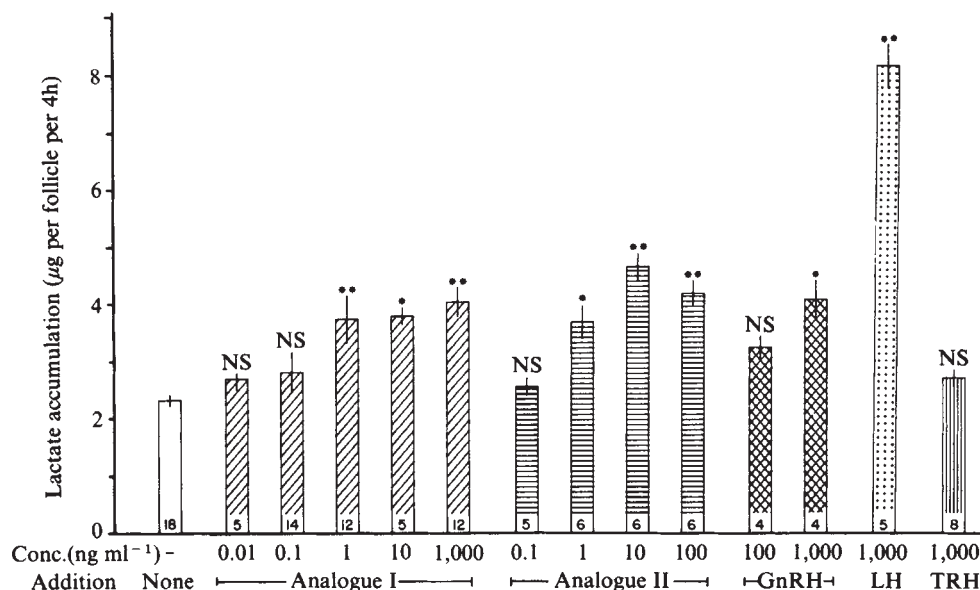


Fig. 2 Effect of LH, TRH and various concentrations of GnRH, analogues I and II on follicular lactate accumulation (μg per follicle) during 4 h incubation. Incubations and lactate measurement were carried out as described in the text. Bar heights represent the means and vertical lines the s.e.m. The number of samples in each group is indicated at the bottom of each bar. Statistical differences between groups were calculated by analysis of variance followed by Student-Newman-Keul's multiple range test. $\square$ * $P < 0.05$; ** $P < 0.01$; NS, not significant.

the stimulatory effect of LH on follicle-enclosed oocytes. It was reported recently that GnRH and an agonist produced meiosis and parthenogenetic-like cleavage when injected repeatedly into hypophysectomized diethylstilboestrol-treated rats¹³. Whether the stimulatory effect by GnRH on oocyte maturation has a physiological role, remains to be determined.

Oocyte maturation is normally triggered by LH, but this hormone seems to stimulate meiosis only indirectly through a primary effect on the follicle cells. Although LH stimulates production of follicular cyclic AMP, prostaglandins, steroids and lactate, none of these substances seem to act as meiosis stimulating factors on the oocyte itself¹⁴. It has been suggested that meiosis is regulated by an intra-follicular inhibitor, the action of which is terminated by LH. An oocyte maturation inhibitor (OMI) has been partially purified from pig follicular fluid and was found to be a peptide with a molecular weight in the order of 2,000 (ref. 15).

The minimal effective dose of GnRH on oocyte maturation in our experiments ($0.1 \mu\text{g ml}^{-1}$) seems to be far above the possible physiological circulating levels of endogenous GnRH¹⁶. However, the possibility has been raised that GnRH might be produced locally in tissues other than the hypothalamus, such as placenta¹⁷ and ovary itself^{4,18}. If this is the case, then the process of oocyte maturation might be determined partly by a balance of locally formed peptides that may be stimulatory (GnRH) and inhibitory (OMI) in nature.

The mechanism by which GnRH and these two analogues stimulate oocyte maturation also remains to be determined.

Clayton *et al.* have shown the presence of specific GnRH receptors in dispersed cells and membrane fractions of luteinized rat ovaries corresponding to a direct action on hormonal stimulation of progesterone and cyclic AMP production^{5,19}. The mechanism of stimulation for these peptides may, however, be different from that for LH, as was shown for the stimulation by GnRH of prostaglandin synthesis in rat granulosa cells⁶. Such a concept seems to be supported by our observation that the maximal stimulation of lactate accumulation, obtained by any test dose of GnRH or its two analogues, was well below the maximal stimulation caused by LH. Further detailed studies of the effect of these peptides on other parameters in our incubation of follicle-enclosed oocytes, such as cyclic AMP accumulation and steroidogenesis, might shed light on this problem.

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Table 2 Specificity of meiosis-inducing effect of GnRH

Compound added	Concentration ($\mu\text{g ml}^{-1}$)	Per cent GVB	n
None	—	12	162
TRH	1	14	44
Oxytocin	0.1	0	20
Somatostatin	0.1	0	19
	1.0	17	42
	10	6	18
GnRH	1	80	45
	10	100	41

Follicle-enclosed oocytes were incubated for 4 h in the presence or absence of various synthetic peptides. The percentage of oocytes with GVB of the total number examined (n) is given.

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Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids

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Anti-inflammatory glucocorticoids inhibit prostaglandin (PG) biosynthesis by preventing arachidonic acid release from phospholipids rather than inhibiting the cyclooxygenase¹⁻¹⁶. As in other cells¹⁷⁻¹⁹, this steroid action depends on receptor occupation and *de novo* protein/RNA biosynthesis¹²⁻¹⁶. We have previously shown in guinea pig perfused lungs and rat peritoneal leukocytes that the effect of steroids on PG generation is mediated by an uncharacterized 'second messenger'^{13,16}. Now, we report that this factor (which we have named 'macroscortin') is an intracellular polypeptide whose release and synthesis are stimulated by steroids. Macroscortin derived from rat peritoneal leukocytes is very similar to that released from guinea pig lungs.

Steroids prevent PG generation by rat peritoneal leukocytes^{14,16,20} and we established that this was due to the release from the cells of a non-dialysable 'second messenger'^{14,16}. In these experiments we found that macroscortin release from

leukocytes was induced by hydrocortisone in 'physiological' concentrations (Fig. 1a) and that the amount released in the presence of a fixed concentration of hydrocortisone was proportional to the number of cells present (Fig. 1b). Analysis of the time course of macroscortin release (Fig. 1c) showed that 30 min after adding hydrocortisone, approximately half the total inhibitory activity was released into the medium. By 90 min most inhibitory activity was extracellular, and by 150 min release was virtually complete. Leukocytes incubated with 1 $\mu\text{g ml}^{-1}$ cycloheximide or without hydrocortisone did not release the inhibitor. Steroids release other factors from leukocytes²¹; was macroscortin synthesized *de novo* in response to the hydrocortisone, was it stored and secreted by a steroid dependent process, or did both mechanisms operate? To answer these questions we lysed untreated rat peritoneal leukocytes by repeated (4 times) freeze-thawing and heated them to 70 °C for 5 min to provide some protection from proteolysis. Dilutions of these lysates possessed high inhibitory activity suggesting that at least some macroscortin was stored in cells. Pretreatment of cells with hydrocortisone reduced intracellular macroscortin by 90%.

In further experiments, groups of 20 male Wistar rats (200–250 g) were treated with dexamethasone (Decadron, Merck; 1.5 mg per kg subcutaneously in the flank) or with saline. The amount of macroscortin contained in a known number of peritoneal leukocytes after collection was determined each hour for 4 h (five rats for each time point) and compared to saline-treated controls. One hour after steroid treatment macroscortin was undetectable in the leukocytes ($n = 3$). After 2 h, the macroscortin content of the cells had returned to control levels ($n = 2$), and increasing amounts (2–3 times control levels) of the material were found 3 and 4 h after treatment ($n = 3$). These experiments indicate that in addition to a steroid-induced depletion of macroscortin, there is a further phase of re-synthesis and that this increases the amount of inhibitor above control levels.

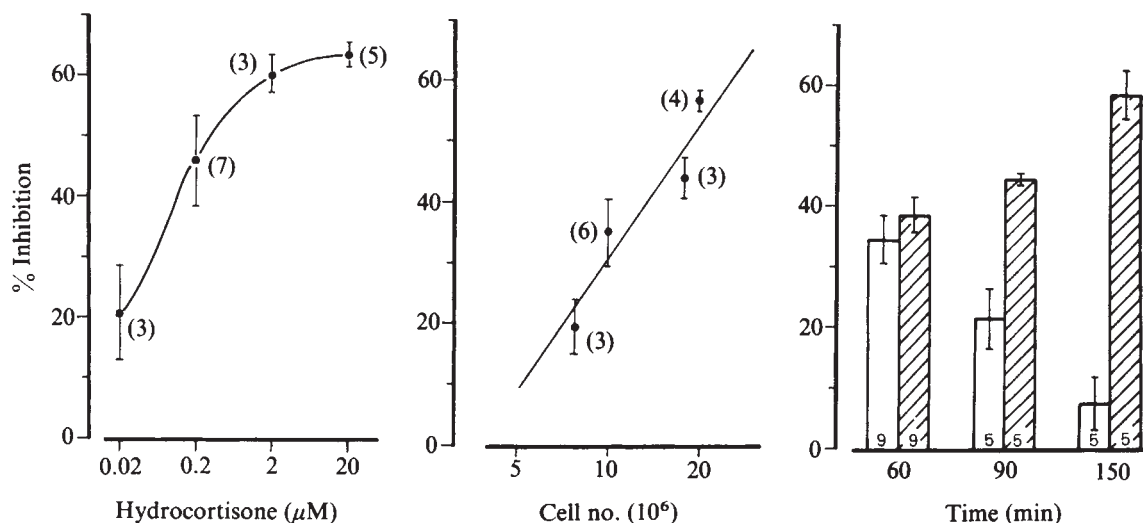


Fig. 1 Male Wistar rats (150–200 g) were killed by exposure to ether. The peritoneal cavity was washed with 20 ml of heparinized Krebs solution and the cell-rich fluid (80% mononucleate and 20% polymorphonucleate) was removed and centrifuged (500 g). The cells from 10–15 animals were pooled and washed twice by resuspending in Krebs solution enriched with bovine serum albumin (BSA, 100 $\mu\text{g ml}^{-1}$). **Release of macroscortin:** The final suspension, usually containing 1×10^7 cells ml^{-1} ('generator leukocytes'), was divided into two samples: hydrocortisone sodium phosphate was added to one sample. No drug was present in the other sample. Both samples were incubated at 37 °C in a metabolic shaker. After incubation, the cells were removed by centrifugation and the supernatants were dialysed overnight against 300 vol Krebs solution at 4 °C. **Assay of macroscortin:** A second pool of freshly collected leukocytes ('test leukocytes') was suspended in Krebs-albumin. A series of 3-ml samples were prepared by mixing 1 ml of cell suspension with 2 ml of dialysed supernatant and incubated for 120 min at 37 °C with killed bacteria (*Bordetella pertussis*) in a ratio of 800–1,000 bacteria per cell. After the incubation the cells were removed by centrifugation and the amount of PGs in the supernatant was determined by bioassay over rat stomach strips using synthetic PGE_2 (Upjohn) as reference standard. Test leukocytes incubated with supernatants collected from steroid-free generator leukocytes released on average 10–12 ng PG (in terms of PGE_2) per 10^6 cells in 120 min. The release was inhibited when the incubation was carried out in presence of dialysed supernatants collected from generator leukocytes treated with hydrocortisone. Results are expressed as mean per cent inhibition ($\pm$ s.e.m.) of the PG formation by test leukocytes exhibited by various supernatants from steroid-treated generator leukocytes, compared with the PG formation in presence of supernatants from steroid-free generator leukocytes. For other experimental details see refs 14, 16. **a**, Macroscortin release by cells incubated for 90 min with increasing concentrations of hydrocortisone (figures in parentheses indicate numbers of experiments). **b**, Relationship between macroscortin release and number of cells (generator leukocytes) incubated with 20 μM hydrocortisone for 90 min. **c**, Time course of macroscortin release. Cells were incubated with hydrocortisone (20 μM) for different times. At the end of incubation the inhibitory activity present in the Krebs solution as well as within the cells were separately determined. After removing the cells by centrifugation, Krebs solution was collected, dialysed and tested as above (hatched columns). The centrifuged cell pellet was resuspended in an equivalent volume of Krebs solution, disrupted by repeated (4 times) freeze-thawing and centrifuged (100,000 g for 60 min). The supernatants were tested for the inhibitory activity as above (open columns). Figures in columns indicate numbers of experiments.

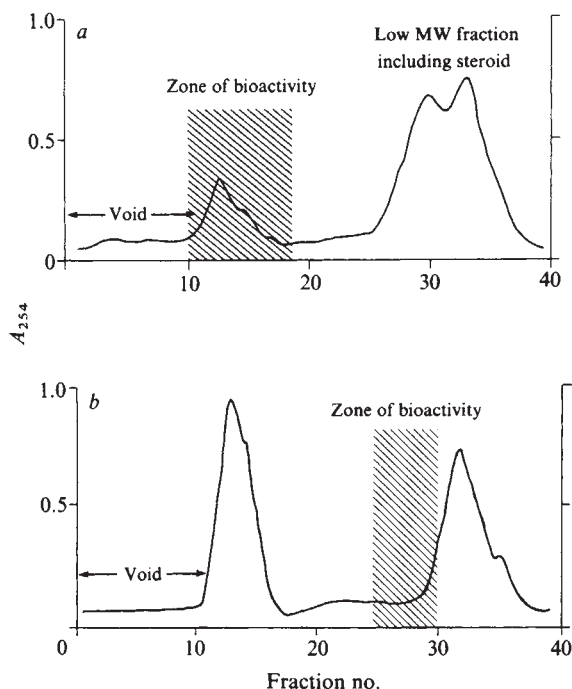


Fig. 2 Partial purification of guinea pig lung macrocortin by Sephadex chromatography. Both columns were 100×1 cm, the equilibrating and eluting buffer used in each case was 0.1% ammonium bicarbonate adjusted to pH 7.5. The flow rate was 0.8 ml min^{-1} and fractions were collected automatically every 5 min. The UV absorbance at 254 nm was continuously measured with a Uvicord flow cell monitor. For isolation of the lung-derived material, batches of the Krebs solution effluent from steroid-treated lungs were processed in an ultrafiltration chamber fitted with a 500 or 1,000 MW filter. The effluent was concentrated to 100–200 ml and then lyophilized. The residual material was dissolved in 10 ml of buffer and applied to the G-25 (medium) column. Fractions were collected and lyophilized. The biological activity was estimated using lungs treated with cycloheximide ($5 \mu\text{g ml}^{-1}$) to prevent the effects of the steroid. *a* Shows the results of such an experiment; several UV absorbing peaks were seen; those associated with fractions 10–20 were compounds with a MW greater than the nominal cut-off point of this gel (in this case, 5,000). The UV absorbing peaks which appeared in fractions 25–40 were low MW compounds including the steroid. In one experiment, in which radioactive dexamethasone was added to the crude effluent, all the radioactivity was subsequently recovered in fractions 30–40. There was no trace of radioactivity in the high MW fractions. In contrast, all of the steroid-like bioactivity was recovered close to the void volume. These preparations were free of steroid and buffer salts and could be conveniently lyophilized. This material was rechromatographed on Sephadex G-50 (medium). In this case however, the biological activity was retained by the column and did not emerge until fractions 21–28. Compounds moving with this mobility would have a MW of $\sim 15,000$. V_e (ml) BSA, 38.5; trypsin inhibitor, 76.5; macrocortin, 84; cytochrome c, 86; dexamethasone, 122.5. Macrocortin production by the lungs was variable. Of all lungs tested, $\sim 15\%$ did not respond to the steroid at all. The inhibitory response to the steroid (thus the production of macrocortin) was enhanced when the glucose concentration in the Krebs solution was increased from 1 g l^{-1} to 2 g l^{-1} . A striking improvement ($\sim$ twofold) in inhibitory activity was observed when a mixture of 21 amino acids (final concentration of each, $\sim 1 \text{ nM}$) was included in the Krebs solution. Leukocyte-derived macrocortin was prepared as follows: sometimes macrocortin released from cells stimulated by hydrocortisone was used as a starting

material, in other instances crude lysates of unstimulated cells were used. In the former case, the medium when separated from the cells was dialysed overnight against 300 vol of Krebs solution at 4°C before column chromatography. In the latter case, the crude, heat-treated lysates were centrifuged at $100,000 \text{ g}$ for 1 h to remove coarse debris and precipitated protein. In either case the resulting material was first passed over a Sephadex G-25 column. As with the lung-derived material, bioactivity was found in the void volume and this was rechromatographed on Sephadex G-50. Again, macrocortin activity was retained by the column and eluted with a position indicating a MW of $\sim 15,000$. The steroid-induced release of macrocortin from leukocyte incubations was sensitive to the glucose concentration (the amount used here (2 g l^{-1}) is essential for complete release). Vigorous gassing of buffers for more than about 5 min apparently prevented release at the cell density used. The amount of macrocortin found in crude lysates of leukocytes was variable and ~ 10 –20% of preparations contained no detectable material, or very small amounts. At the moment, we cannot gauge the influence of endogenous steroids on the macrocortin content of leukocytes and this could well be a source of variation.

Partially purified macrocortin has been isolated from two sources—the effluent from steroid-stimulated guinea pig lungs, and rat peritoneal leukocytes—see Fig. 2 legend for method. Macrocortin from both sources has an apparent molecular weight (MW) of 15,000 and also has a number of properties in common: both preparations are stable at 70°C for up to 30 min but are destroyed by boiling. Acidification of the factor to pH 2 for 20 min resulted in less than 15% loss of activity. Macrocortin seems to be a protein because lung-derived material lost substantial (40–70%) activity after being stirred overnight at 4°C with trypsin ($10 \mu\text{g ml}^{-1}$) or papain ($10 \mu\text{g ml}^{-1}$). The biological activity of partially purified (ex G50) leukocyte-derived macrocortin was also reduced (60–70%) by incubation for 1 h at 37°C with 2.0 U of immobilized trypsin (Pierce).

Simple experiments with crude lung or leukocyte macrocortin demonstrated a considerable similarity in their biological activity: phagocytosing rat leukocytes produced less PGs when incubated in effluent from steroid-treated lungs than in effluent from control lungs (3.86 ± 0.53 and $5.63 \pm 0.69 \text{ ng PGE}_2$ per 10^6 cells; $n = 7$). Krebs solution, in which leukocytes and steroid had been incubated together, inhibited PG synthesis by fresh leukocytes and thromboxane release from the lung¹³. Macrocortin from leukocyte lysates inhibited strongly in both systems. In each type of experiment, boiling abolished, and arachidonate reversed, the inhibition indicating that the inhibitor was heat labile and did not act at the cyclo-oxygenase level. The inhibition was cycloheximide-resistant and could not therefore be due to residual steroid.

Leukocyte-derived macrocortin also inhibited phospholipase activity in the lung as estimated by a radiochemical technique (see Fig. 3). The inhibitory activity was of rapid onset and was abolished by boiling.

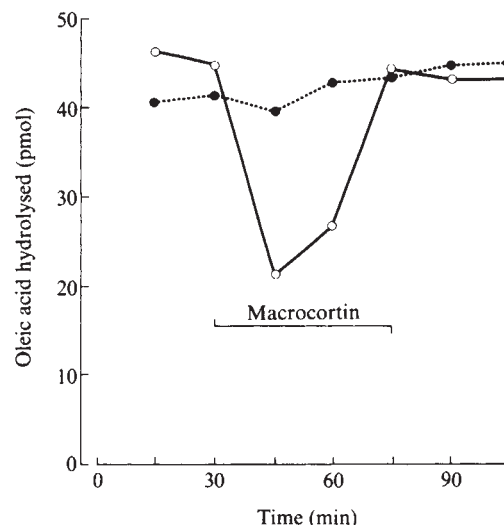


Fig. 3 Inhibition of guinea pig lung phospholipase A_2 activity by leukocyte-derived macrocortin. The assay depends on a specifically labelled molecule 1-palmitoyl, 2- ^3H -oleic phosphatidylcholine. The rationale of the assay, preparation of the isotope and further methodology are described in ref. 11. Briefly, a mixture of the tritiated phosphatidylcholine and ^{14}C -labelled oleic acid ($0.1 \mu\text{Ci}$ total), dissolved in Tris buffer (pH 7.5), was injected into the Krebs solution entering the pulmonary artery. The effluent was collected for 5 min, 5 ml of Tris buffer (pH 7.5; 100 mM) was added to stabilize the pH, and the labelled fatty acids were extracted into 5 ml hexane by 1 min vortex mixing. An aliquot of the hexane was transferred into scintillation vials and the solvent evaporated with a gentle stream of nitrogen. Liberation of the ^3H -oleic acid from the phosphatide by phospholipase A_2 increases the $^3\text{H}/^{14}\text{C}$ ratio of the extracted fatty acids, enabling percentage hydrolysis to be estimated. The ratio of the two isotopes in a small aliquot of the aqueous solution was also determined: this gave a value for the 'total hydrolysis' and the actual hydrolysis was then calculated. $\circ$, Normal macrocortin (ex G25); $\bullet$, boiled material. Lungs had been perfused for 1 h at time '0 min' on the graph. The inhibitory activity was of rapid onset without the latency seen with the corticosteroids.

Arachidonate-derived products such as PGs²² and 12-hydroxy-5, 8, 10, 14 eicosatetraenoic acid²³ (12-HETE) are involved in the pathogenesis of inflammation. The aspirin-like drugs block the cyclooxygenase²⁴⁻²⁶ and this probably explains their therapeutic efficacy. Steroids are without activity on the cyclooxygenase enzyme but potentially inhibit PG biosynthesis in viable cells. An important link was established between phospholipase inhibition and steroid action by Gryglewski's group³ and Danon and Assouline were the first to propose that this was due to an action on the genome¹². We took this concept further and provided indirect evidence for a second messenger which mediates the anti-phospholipase effects of steroids^{13,16}. Here we provide the first direct evidence for such a factor (macroscortin) and have determined that it is a polypeptide of approximately

15,000 MW. In leukocytes steroids cause both a release and re-synthesis of macroscortin—both effects are blocked by inhibitors of RNA/protein synthesis.

Most aspirin-like drugs block only the cyclooxygenase pathway and may potentiate the formation of other arachidonate-derived substances, such as the chemotactic 12-HETE²⁷ and the bronchoconstrictor slow-reacting substance of anaphylaxis²⁸. The steroids differ in this respect and block the formation of all products as they restrict substrate (arachidonate) availability. This may explain why they are able to block cell migration and allergic bronchoconstriction whereas the aspirin-like drugs cannot. In view of the work reported here, we propose that the most likely effector of steroid action on the arachidonate cascade is macroscortin.

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Thyrotropin-releasing hormone—CNS action to stimulate gastric acid secretion

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Much physiological and pharmacological evidence has accumulated to suggest that the autonomic nervous system has an important role in the peripheral modulation of gastric secretion¹, although the neurochemical mediators in the brain which initiate or modulate autonomic input are poorly understood¹⁻⁴. Recently, the demonstration that some oligopeptides present in mammalian brain act in the central nervous system (CNS) to influence profoundly glucoregulation⁵, thermoregulation^{6,7}, blood pressure^{8,9}, sympathetic outflow⁹⁻¹¹, muscular activity of gut^{12,13} and stress-induced gastric haemorrhagic lesions¹⁴ have led us to examine a possible role for some of these endogenous brain oligopeptides as chemical messengers involved in the CNS modulation of gastric secretion. We report here that thyrotropin-releasing hormone (TRH) acts within the CNS to elicit a vagus-dependent stimulation of gastric acid secretion.

In this study we used male Sprague-Dawley CD rats (250–300 g) maintained *ad libitum* on Purina Laboratory Chow and tap water, and housed in conditions of controlled temperature and illumination (from 06:00 to 22:00 h). All experiments were performed according to the same time schedule in 24-h food-deprived rats with free access to water up to the beginning of treatment. Saline or test materials were injected intracisternally (i.c.) or intravenously (i.v.) via the jugular vein under light ether anaesthesia, then the pylorus portion of the stomach was ligated. In some experiments, immediately before peptide injection and pylorus ligation, subdiaphragmatic vagotomy or sham operation was performed using the general method described by Martin *et al.*¹⁵. Conscious animals were decapitated at various intervals following pylorus ligation and gastric content was collected.

Gastric volume and pH were measured and samples (0.5 or 1 ml) analysed for gastric acidity by titration with 0.01 M sodium hydroxide to a pH of 7.0.

Peptides, synthesized by Dr J. Rivier using solid phase methodology¹⁶, and atropine sulphate were freshly dissolved in 0.9% saline before use and injected i.c. in 10 µl or i.v. in 0.2 ml volume. Following analysis of variance, differences between groups were determined by the multiple range test of Dunnett and Duncan using the computer program EXBIOL.

TRH given i.c. induced a marked elevation in the volume, titratable acid concentration and output of gastric secretion (Table 1). The time course of gastric response to TRH (expressed as percentage of changes compared with control decapitated at the same time period and taken as 100% change) indicated that the stimulatory effect of TRH reached a maximum within

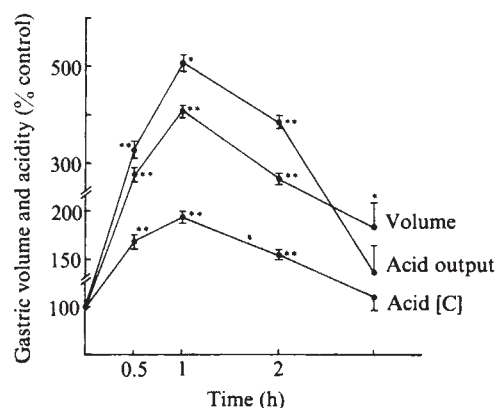


Fig. 1 Temporal effect of TRH (1 µg, i.c.) on gastric secretion. Twenty-four-hour fasted rats were injected with saline or TRH, then the pylorus was ligated and groups of saline and TRH-treated rats were decapitated at 30 min, 1, 2, or 3 h post-treatment. Each curve represents changes in gastric secretion induced by TRH injection, expressed as percentage compared with control groups taken for each time period as 100% change. * $P < 0.05$; ** $P < 0.01$ as compared with saline-treated group decapitated at the same time period post-injection.

Table 1 Gastric secretory response to i.c. injection of TRH and TRH analogues

Peptide	No. of rats	Volume (ml per rat)	pH	Gastric secretion	
				Titratable acidity	
				Concentration (mequiv. l ⁻¹)	Output (μequiv.)
Saline	7	2.1 ± 0.5	1.87 ± 0.09	61 ± 9	154 ± 65
TRH	7	5.3 ± 1.0*	1.51 ± 0.05†	95 ± 9†	551 ± 134*
[³ Me-His ²]-TRH	7	4.9 ± 0.8*	1.51 ± 0.03†	88 ± 8†	464 ± 111†
[¹ Me-His ²]-TRH	8	1.6 ± 0.4	1.71 ± 0.11	69 ± 7	130 ± 44

24-h fasted rats under light ether anaesthesia were injected i.c. with saline (10 μl) or peptides (1 μg per 10 μl) and the pylorus was ligated. After 2 h, the animals were decapitated and gastric secretion collected.

* $P < 0.001$; † $P < 0.05$ as compared with saline-treated rats.

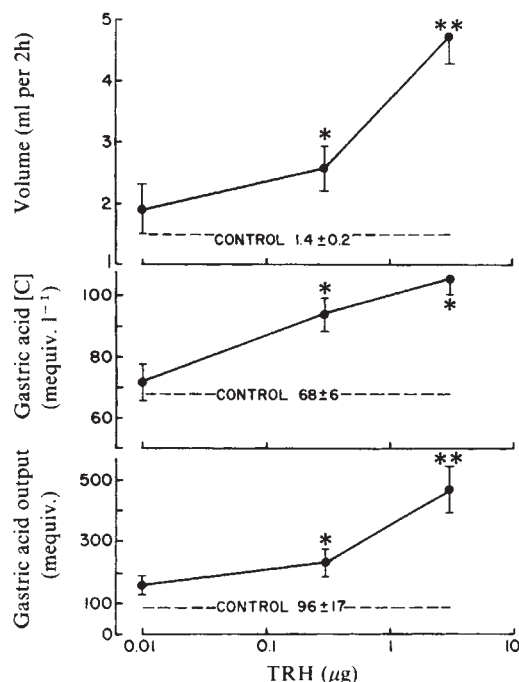


Fig. 2 Dose-dependent stimulatory effect of TRH given i.c. on gastric secretion. Twenty-four-hour fasted rats were injected i.c. with saline (control values) or various doses of TRH and the pylorus was ligated. Two h later, rats were killed and gastric secretion collected. Each point represents the mean ± s.e.m. of 5–12 animals. * $P < 0.05$; ** $P < 0.01$ as compared with control.

1 h, thereafter it returned to the level of control group (Fig. 1). A significant dose-related stimulatory effect is observed following i.c. injection of TRH in doses ranging over 0.3–3 μg as measured 2 h after peptide injection and pylorus ligation (Fig. 2). TRH given i.v. at 10 or 100 μg dose per animal exerted no effect on gastric secretion (data not shown). Using a different experimental design, Morley *et al.* have observed in dogs that TRH inhibited gastric acid secretion stimulated by tetragastrin¹⁷ for the first 15 min. The activity of TRH given i.c. and its ineffectiveness given i.v. strongly support the concept that TRH initially acts through the brain to elicit the stimulation of gastric secretion. The tripeptide has already been shown to have a broad spectrum of CNS-mediated actions unrelated to its neuroendocrine activity, including modification of behavioural¹⁸ and hormonal^{19–21} effects of narcotics, or biogenic amines, alteration of thermoregulation²², blood pressure²³, food intake²⁴ and muscular activity of gut^{12,13}. The stimulatory effect of TRH on gastric secretion appears long-acting, dose-depen-

dent, reversible and specific. The specificity is demonstrated by the inactivity of TRH analogue [¹Me-His²]-TRH (Table 1) which likewise has been found ineffective in other bioassay systems^{19,22,25}, whereas the active analogue [³Me-His²]-TRH (ref. 25) mimicked the stimulatory effect of TRH on gastric secretion. Antimuscarinic compounds have been shown to antagonize several CNS-mediated effects of TRH including those on eye pupil¹⁸ and gut muscular activity^{12,13}. The effectiveness of vagotomy (data not shown) or atropine (Table 2) in inhibiting the stimulatory effect of TRH on gastric secretion supports the concept that vagus-dependent cholinergic mechanisms could participate in the expression of the gastric response to TRH. However, the exact neuroanatomic site, as well as the CNS and peripheral neuronal/humoral circuits involved, remain to be further identified. On the basis of stimulation and lesion experiments, the hypothalamus (mainly the lateral and ventromedial regions) has been demonstrated in rats to participate in brain control of gastric secretion^{26,27}. The fact that high concentrations of TRH are present in the hypothalamus²⁸ supports the possibility that endogenous TRH may have physiological significance as a CNS chemical messenger involved in brain modulation of gastric secretion.

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Table 2 Blockade by atropine of TRH-induced stimulation of gastric secretion

Group	Treatment	No. of rats	Volume (ml per rat)	Gastric secretion	
				Titratable acidity	
				Concentration (mequiv. l ⁻¹)	Output (μequiv.)
1	Saline + saline	13	1.1 ± 0.2	61 ± 10	88 ± 2
2	Saline + TRH	9	3.3 ± 0.3*	107 ± 6*	364 ± 35*
3	Atropine + saline	5	0.3 ± 0.1†	30 ± 10†	18 ± 10†
4	Atropine + TRH	5	0.4 ± 0.2† (*)	28 ± 5† (*)	8 ± 4† (*)

24-h fasted rats under light ether anaesthesia were injected i.v. with saline (0.2 ml) or atropine (2 mg per kg) and i.c. with saline (10 μl) or TRH (1 μg 10 μl⁻¹) and the pylorus was ligated. One h later, the animals were decapitated and gastric secretion collected. * $P < 0.01$; † $P < 0.05$ as compared with group 1 and in parentheses with group 2.

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Evidence that substance P is a neurotransmitter in the myenteric plexus

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Substance P (SP) is an undecapeptide originally isolated from the gut¹ and since shown to occur within neurones in several parts of the peripheral^{2,3} and central⁴ nervous systems. Immunohistochemical studies indicate an exceedingly dense network of SP-containing nerves within the myenteric plexus of the guinea pig ileum^{5,6}. These nerves are intrinsic to the gut wall^{5,7} and can release SP to contract the longitudinal muscle layer^{8,9}. We have previously shown^{10,11} that SP directly depolarizes myenteric neurones and that this depolarization has a time course and ionic mechanism similar to the slow excitatory postsynaptic potential (e.p.s.p.) which can be produced by electrical stimulation of presynaptic nerves within the myenteric ganglia. We wondered whether SP might mediate this slow synaptic potential¹⁰. We report here that the SP depolarization and the slow e.p.s.p. are reversibly depressed by chymotrypsin, an enzyme which degrades SP, although the responses to acetylcholine, serotonin and an unknown hyperpolarizing transmitter are unaffected. The results provide direct evidence that a peptide can mediate chemical transmission between neurones in the mammalian nervous system.

Intracellular recordings were made from neurones in myenteric ganglia which had been dissected from the ilea of adult guinea pigs¹². These ganglia were perfused *in vitro* with warm

(37 °C) Krebs solution to which known concentrations of SP or chymotrypsin could be added. The neurones could be seen using Nomarski optics and this allowed placement of the tips of iontophoresis electrodes containing SP or serotonin close to the soma membrane of the impaled cell^{11,12}. Focal stimulation of the ganglion surface evoked fast cholinergic synaptic potentials in one class of cells (type 1 (ref. 12), S cells¹³) and sometimes caused a much slower depolarization¹⁴ in both types of cell (also type 2 (ref. 12), AH cells¹³). Perfusion of SP depolarized both types of myenteric neurones and also reversibly blocked the slow e.p.s.p. and the depolarizing response to SP iontophoresis; it did not block the fast cholinergic e.p.s.p. This finding at least suggested that the perfused SP may desensitize the myenteric neurones to the transmitter released by nerve stimulation.

Perfusion of the preparation with chymotrypsin (200 µg ml⁻¹) reversibly abolished the responses to SP applied by either perfusion (Fig. 1a) or iontophoresis (Fig. 2). The response to SP iontophoresis could be restored in the presence of chymotrypsin by increasing the amplitude or duration of the ejecting current, arguing against a destruction or inactivation of postsynaptic sites and in favour of enzymatic degradation of SP between the time of its release from the iontophoresis pipette and its action on the cell membrane. Chymotrypsin (200 µg ml⁻¹) reversibly abolished the slow e.p.s.p. (Fig. 1b). When the synaptic potential was depressed by chymotrypsin, it could sometimes be restored by increasing the frequency or duration of the stimulating train of pulses. Chymotrypsin usually did not change the membrane potential, input resistance, action potentials or fast e.p.s.p.; nor did it affect the slow hyperpolarizing synaptic potentials which were occasionally observed in response to nerve stimulation¹⁴. Higher concentrations of chymotrypsin (300–1,000 µg ml⁻¹) depolarized myenteric neurones, often irreversibly. Chymotrypsin (200 µg ml⁻¹) did not block either the depolarizing or the hyperpolarizing responses to iontophoretic application of serotonin (Fig. 2). Carboxypeptidase (200 µg ml⁻¹), which does not cleave the amidated C terminal of SP (ref. 15), had no effect on membrane potential, the fast e.p.s.p., the slow e.p.s.p. and the

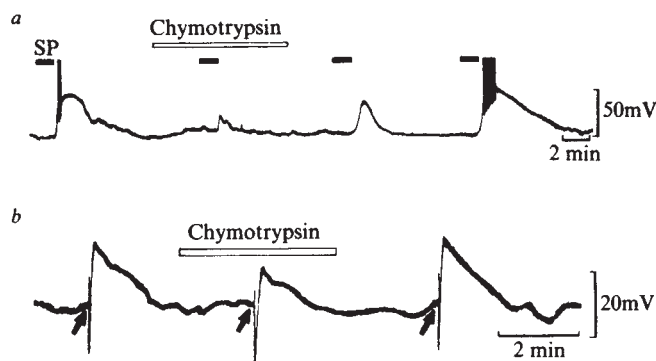


Fig. 1 Recordings of membrane potential from two myenteric neurones. *a*, Substance P depolarization is abolished by chymotrypsin. The perfusion solution contained SP (100 nM) during the four periods (90 s) indicated by the solid bars. The perfusing solution also contained chymotrypsin (200 µg ml⁻¹) where indicated by the open bar. The apparent delay in the onset of the depolarizing action of SP is due entirely to the time taken for the changed solution to flow through the perfusion system before entering the bath (80 s). SP caused a prominent depolarization which initiated action potentials. Chymotrypsin reversibly prevented this action. *b*, Slow e.p.s.p. is depressed by chymotrypsin. The surface of the myenteric ganglion was stimulated with a focal stimulating electrode (10 Hz, 3 s) at the times indicated by the arrows. This caused a short-lasting hyperpolarization followed by a prolonged depolarization (slow e.p.s.p.). Perfusion of chymotrypsin reversibly depressed the slow e.p.s.p. without changing membrane potential or the hyperpolarizing response (which is, presumably, due to a different transmitter).

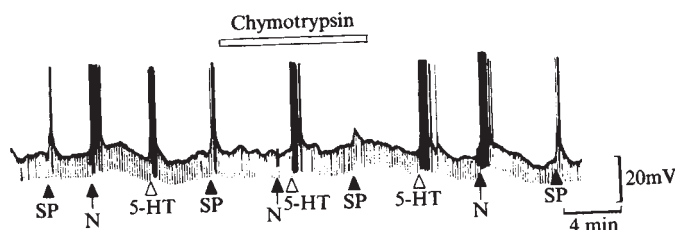


Fig. 2 Membrane potential of a myenteric neurone. Downward deflections are anelectrotonic potentials caused by constant current pulses. SP (100 nA, 5s) and serotonin (5-HT, 20 nA, 10s) were applied at the times indicated by $\blacktriangle$ and $\triangle$, respectively. Presynaptic nerves were stimulated (30 Hz, 3 s) at the times indicated by the arrows (N). Both SP and serotonin mimicked the effects of nerve stimulation in this neurone, causing it to depolarize and fire action potentials (full amplitude not recorded). Perfusion with chymotrypsin ($200 \mu\text{g ml}^{-1}$) did not change membrane potential or resistance but abolished the response to nerve stimulation and greatly reduced the response to SP iontophoresis. The response to serotonin iontophoresis was not depressed.

depolarizing responses to iontophoretic application of either SP or serotonin. Higher concentrations of carboxypeptidase depolarized myenteric neurones, often irreversibly.

In the present experiments, chymotrypsin did not depress the serotonin response in the same cell in which it depressed the slow e.p.s.p. It seems unlikely that serotonin makes a major contribution to the slow e.p.s.p., as has been proposed¹⁶. It is undetectable in the myenteric plexus of the adult guinea pig ileum without precursor loading and the proportion of cells which contain aromatic amino acid decarboxylase is very low. Furthermore, serotonin either depolarizes or hyperpolarizes myenteric neurones¹⁷, and a substantial proportion of those which are hyperpolarized have only depolarizing slow e.p.s.ps (Johnson, Y.K., K.M. and R.A.N., in preparation).

The results indicate that a chymotrypsin-sensitive peptide mediates the slow depolarizing potential; the great density of nerve terminals which contain SP make this peptide a likely candidate. At other sites in the mammalian nervous system, evidence for 'peptidergic' transmission remains largely inferential. Enzymatic degradation of released peptide transmitters, although lacking the specificity of pharmacological antagonists, may offer an as yet untested means of establishing the functional role of peptides within the nervous system.

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Neurotransmitter turnover in rat striatum is correlated with morphine self-administration

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Drugs of abuse probably exert their reinforcing effects through 'reward' pathways in the central nervous system (CNS)^{1,2}. Neuronal systems mediating opiate reinforcement have been investigated using pharmacological and electrolytic lesion procedures. Drugs that interfere with catecholaminergic³⁻⁶ and cholinergic⁷ neuronal activity decrease intravenous (i.v.) morphine self-administration in monkeys and rats. Electrolytic lesion procedures in rats have demonstrated that the medial forebrain bundle⁸ and caudate nucleus⁹ are important in maintaining i.v. morphine self-administration. We have now carried out a direct investigation of striatal (caudate nucleus, putamen and globus pallidus) neuronal systems. We show here that striatal catecholaminergic systems are important in mediating opiate reinforcement, and present direct evidence for the involvement of neurotransmitter systems in morphine reward.

The turnover rates of dopamine (DA), noradrenaline (NA), aspartate (Asp), glutamate (Glu), γ -aminobutyric acid (GABA) and glycine (Gly) were determined in rats self-administering morphine i.v. (condition 1) and in yoked-morphine infused (condition 2) and yoked-vehicle infused (condition 3) littermates. This design was chosen to elucidate the neurochemical effects of the drug alone (conditions 2 and 3) and the effects of the drug self-administration milieu (conditions 1 and 2).

Eleven litters of three (conditions 1-3) adult male Fisher 344 rats were implanted with chronic jugular catheters using a previously described procedure^{10,11} and housed together in a large experimental chamber in three separate self-administration cages. Two rats in each litter were made physically dependent over an 8-day period by automatic hourly infusions of morphine in increasing concentrations (2 days each of 1.25, 2.5, 5.0 and 10.0 mg per kg); the third rat received yoked-vehicle (saline) infusions. Each infusion (morphine or saline) was paired with a tone and light stimulus of 30 s duration. Levers were then introduced and one of the physically dependent rats was allowed to self-administer morphine i.v. (10 mg per kg in 0.1 ml saline) by lever pressing. This animal had 24 h access to morphine from that time and could take an infusion on demand by fulfilling the response requirement, which was initially one

Table 1 Content of dopamine, noradrenaline, serotonin (5-HT), aspartate, glutamate, GABA and glycine concurrently measured in the striatum of rats i.v. self-administering morphine and in yoked-morphine and yoked-vehicle infused littermates

	Content				Content			
	pmol per mg protein				nmol per mg protein			
	DA	NA	5-HT		Asp	Glu	GABA	Gly
Morphine self-administration	524.5	34.2	45.6		20.1	86.8	25.3	6.0
Yoked-morphine infused	± 77.5	± 9.6	± 5.9		± 3.6	± 10.5	± 4.9	± 1.3
Yoked-vehicle infused	524.1	35.5	44.9		22.2	93.8	29.8	6.8
	± 76.5	± 7.4	± 5.7		± 3.4	± 10.3	± 5.8	± 1.4
	549.8	36.1	45.8		21.4	88.0	27.1	6.5
	± 85.0	± 8.2	± 5.0		± 4.3	± 8.0	± 7.1	± 1.5

Values are means $\pm$ s.d. for $n = 11$ in each treatment condition. Student's t -test for differences between means showed no significant changes.

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lever press. Eventually, 10 lever presses were required for each injection (fixed ratio 10 schedule of reinforcement). The yoked-morphine and yoked-vehicle injected animals in each litter received simultaneous infusions whenever the self-administration animal earned an infusion. Lever presses from these yoked littermates were recorded. However, such responses were infrequent.

After stable baselines of self-administration had been obtained, the cumulative records for the 3 days before death were used to determine an average inter-injection interval for each litter. On the day of pulse-labelling, at 60 ($n = 5$ litters) and 90 min ($n = 6$ litters) before a predicted injection, 0.5 mCi [^3H]tryptophan (NEN, specific activity 7.1 Ci mmol $^{-1}$), 1.0 mCi [$^2,6\text{-}^3\text{H}$]tyrosine (Amersham Searle, specific activity 34 Ci mmol $^{-1}$) and 0.2 mCi D-[^{14}C]glucose (ICN, specific activity 210 mCi mmol $^{-1}$) were injected through the jugular catheter. The three littermates were killed at the calculated injection time by total immersion in liquid nitrogen. The heads were allowed to warm to -20°C , the brain removed, cut into 1-mm coronal sections, and the striata discretely removed from the frozen sections. The striata were individually pulverized in a stainless steel mortar in liquid nitrogen and the content, specific radioactivity and turnover values for DA, NA, Asp, Glu, GABA and Gly were concurrently determined using previously described procedures¹²⁻¹⁴.

Stable morphine self-administration usually developed within 3-4 weeks (27 ± 16 days) (all values are mean $\pm$ s.d.), the average inter-injection interval being 122 ± 35 min for the 11 self-administering animals. During the pulse-label interval, neither morphine nor vehicle was delivered to the litter even if the self-administering animal responded appropriately for an injection. However, the stimulus light and tone were presented, resulting in conditioned morphine-like stereotypical behaviour (weaving, bobbing, circling) in both morphine-dependent rats in each litter during the pulse-label interval. Self-administering animals responded for an average of 5 ± 4 injections (~ 50 responses) during the pulse interval, with most responses occurring near the predicted injection time. These non-reinforced lever presses were a small percentage of the total motor activity occurring during the pulse interval so that neurochemical differences between the morphine self-administering rat and the yoked-morphine injected animals would be unlikely to result from the purely motor effects of these non-reinforced lever presses but may well partially result from the motivational effects of non-reinforcement (extinction).

The striatal content of the putative neurotransmitters and amino acids did not differ in any of the treatment conditions (Table 1), whereas turnover rates were very different in the yoked-morphine infused and self-administering animals (Table 2). Passive morphine infusions alone (yoked-morphine group compared with yoked-saline infused group) resulted in an increase in the turnover rates of DA, Glu, Gly and GABA and a decrease in NA utilization. The effects of the contingent administration of morphine were sometimes even greater. Turnover rates of DA, NA, Asp, Glu and GABA were almost twice and Gly almost half as high in the self-administering rats as in the yoked-morphine infused animals.

The differences between the self-administering and yoked-morphine infused animals are important for several reasons. First, these data demonstrate that the contingent effects of drug self-administration behaviour result in changes in the utilization rates of putative neurotransmitters in the striatum that are sometimes greater than the effect of the drug itself. The interaction between the drug and the environmental context of its abuse, as reflected in this study by the conditions of contingent compared with non-contingent administration, is an important factor in abuse potential that has been recognized in the clinical setting. Humans receiving opiates in a passive fashion (for example, hospitalized individuals receiving opiates for pain) are less likely to self-administer the drug after it has been withdrawn or to engage in drug-seeking behaviour than are addicts who self-administer and are subsequently withdrawn. Self-adminis-

Table 2 Turnover rates for dopamine, noradrenaline, aspartate, glutamate, GABA and glycine concurrently measured in the striatum of rats i.v. self-administering morphine and in yoked-morphine and yoked-vehicle infused littermates

	pmol per mg protein per h		Turnover rates nmol per mg protein per h			
	DA	NA	Asp	Glu	GABA	Gly
Morphine	287.0*	21.6*	32.2*	112.8*	33.9*	1.1*
self-administration	± 42.6	± 6.3	± 5.7	± 13.7	± 6.6	± 0.2
Yoked-morphine	117.8*	10.8*	17.8†	55.7*	12.9*	3.0*
infused	± 17.6	± 2.2	± 2.7	± 6.1	± 2.5	± 0.6
Yoked-vehicle	46.6	45.5	13.9	35.9	3.2	1.5
infused	± 7.7	± 10.3	± 2.8	± 3.3	± 0.9	± 0.3

Turnover rates were measured after administration of ^3H -tryptophan, ^3H -tyrosine and ^{14}C -glucose. Values are means $\pm$ s.d. for $n = 11$ in each treatment group and were calculated as the product of the fractional rate constants (k) and the content values from Table 1. Turnover_A = k (content_A). Any measure of neurotransmitter turnover is an approximation, but represents relative rates of metabolism of transmitters and amino acids. Thus, two time points on the linear decay portion of temporal specific activity plots were used to calculate apparent fractional rate constants based on assumptions such as steady state and one open pool, which are obviously not entirely valid and consistent with current concepts of putative neurotransmitter compartmentation. Nevertheless, to obtain a measure of transmitter utilization, the fractional rate constants were calculated as $k = \ln 2 / t_{1/2}$, where $\ln 2 = 0.693$ and the $t_{1/2}$ is obtained from a semilogarithmic plot of the specific radioactivities determined at 60 ($n = 5$ litters) and 90 ($n = 6$ litters) min after i.v. injection of 0.5 mCi of ^3H -tryptophan, 1.0 mCi ^3H -tyrosine and 0.2 mCi ^{14}C -glucose. The turnover rate of serotonin was too low for reliable measurement.

* $P < 0.001$; † $P < 0.01$: the significance of the difference between means as determined with Student's t -test. The self-administration group was compared with the yoked-morphine infused group (effects of self-administration) and the latter was compared with the yoked-vehicle infused group (effects of morphine).

tration sometimes results in greater changes in striatal neurotransmitter turnover than does passive infusion of the drug into yoked littermates, and seems to have marked neurochemical consequences. Second, these differences demonstrate for the first time a neurochemical correlate of drug self-administration behaviour. Our data indicate that the milieu in which a drug is administered cannot be ignored in basic studies of drug action.

The role of CNS neurotransmitters in opiate action has been extensively investigated¹²⁻¹⁹. Also, several lines of evidence indicate that opiate reinforcement may be mediated by central reward pathways²⁰⁻²³. This is further supported by the increase in DA turnover in the striatum of self-administering animals seen in the present study. Furthermore, changes in the turnover rates of the amino acids are consistent with their role as putative transmitters released at a large number of CNS synapses²⁴. The interaction of multiple systems during contingent opiate self-administration is now being assessed.

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Supraoptic neurones of rat hypothalamus are osmosensitive

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It has been found that cells in the region of the supraoptic nucleus (SON) of the hypothalamus are sensitive to osmotic stimuli in a physiological range, and have studied the mechanism by which the osmotic sensitivity arises. An *in vitro* hypothalamic brain slice preparation has been used to make intracellular recordings from the SON. Cells lying in the SON respond to small increases ($9\text{--}40\text{ mosmol l}^{-1}$) in the osmolarity of their external environment with a marked increase in firing rate. They respond to NaCl and mannitol although not to glucose. The osmotic sensitivity of SON neurones has a complex origin at both a pre- and a postsynaptic level, being composed first of depolarization of the primary SON neurone by an increase of extracellular osmolarity and second by an increased rate of occurrence of excitatory synaptic events which markedly augment firing rate. These results are consistent with Jewell and Verney's suggestion^{1,2} that osmoreception in the mammalian brain occurs in the region of the anterior hypothalamus, and extends this localization by indicating that the SON neurones are themselves both directly osmosensitive and part of an osmoreceptive complex.

Hypothalamic neurones in the mammalian SON are known to control the secretion of oxytocin and vasopressin from the posterior pituitary into the systemic circulation. Jewell and Verney² originally proposed that the osmoreceptors regulating vasopressin secretion lay outside the blood–brain barrier in the region of the anterior hypothalamus, and they suggested the SON as one possible site at which osmoreception might occur. Consistent with this, Sladek and Knigge³ concluded from work with hypothalamo-neurohypophysial explants that the osmoreceptors lay near to the SON. This proposal, however, has generated considerable controversy, centering on three points. First, several workers have disagreed as to whether neurosecretory cells in the SON are themselves osmoreceptors or whether they receive synaptic input from osmoreceptors more distant from the SON^{4–6}. Second, is the osmoreceptor really osmotically sensitive, or can the responses observed *in vivo* be accounted for by a sodium receptor^{4,5,7–9}? A final area where conflicting results have emerged has been generated by recent work using extracellular recording from the slice preparation by Hattton *et al.*¹⁰ and Wakerley and colleagues^{11,12}, which found little spontaneous activity and little or no osmosensitivity in the SON, results which conflicted in turn with Leng's finding⁶ that direct 'microtap' application of hyperosmolar NaCl *in vivo* excited SON neurones directly. These controversies were thus examined with the potentially powerful *in vitro* slice technique. This technique has several advantages, including: (1) anaesthetics are unnecessary, (2) the preparation is continuously superfused so that known concentrations of substances of interest can be applied, (3) the SON can be identified unambiguously by its proximity to the optic chiasm, (4) the blood–brain barrier is effectively absent, and (5) stable, long-term intracellular records can be obtained.

Coronal or sagittal slices, 300–400 μm thick, of rat hypothalamus were prepared on a vibratome and maintained in oxygenated Yamamoto¹⁴ medium (pH 7.5) at 37 °C. The slices were supported on lens tissue/acrylic mesh and O_2/CO_2 (95/5%) moistened with medium blown over them. Perfusion from beneath was accomplished with an LKB peristaltic pump operated at 0.6–1.0 ml min^{-1} in a chamber volume of about 1.5 ml. Renewal of medium in the chamber was 95% complete

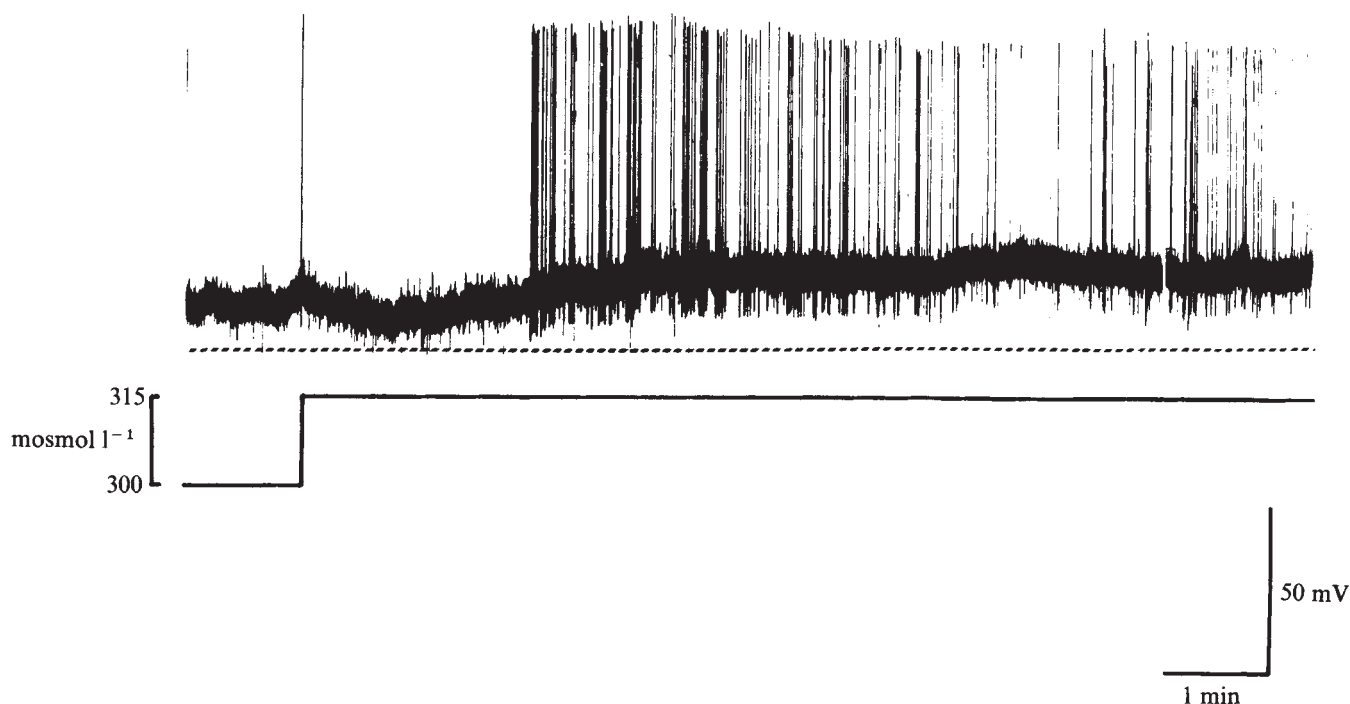
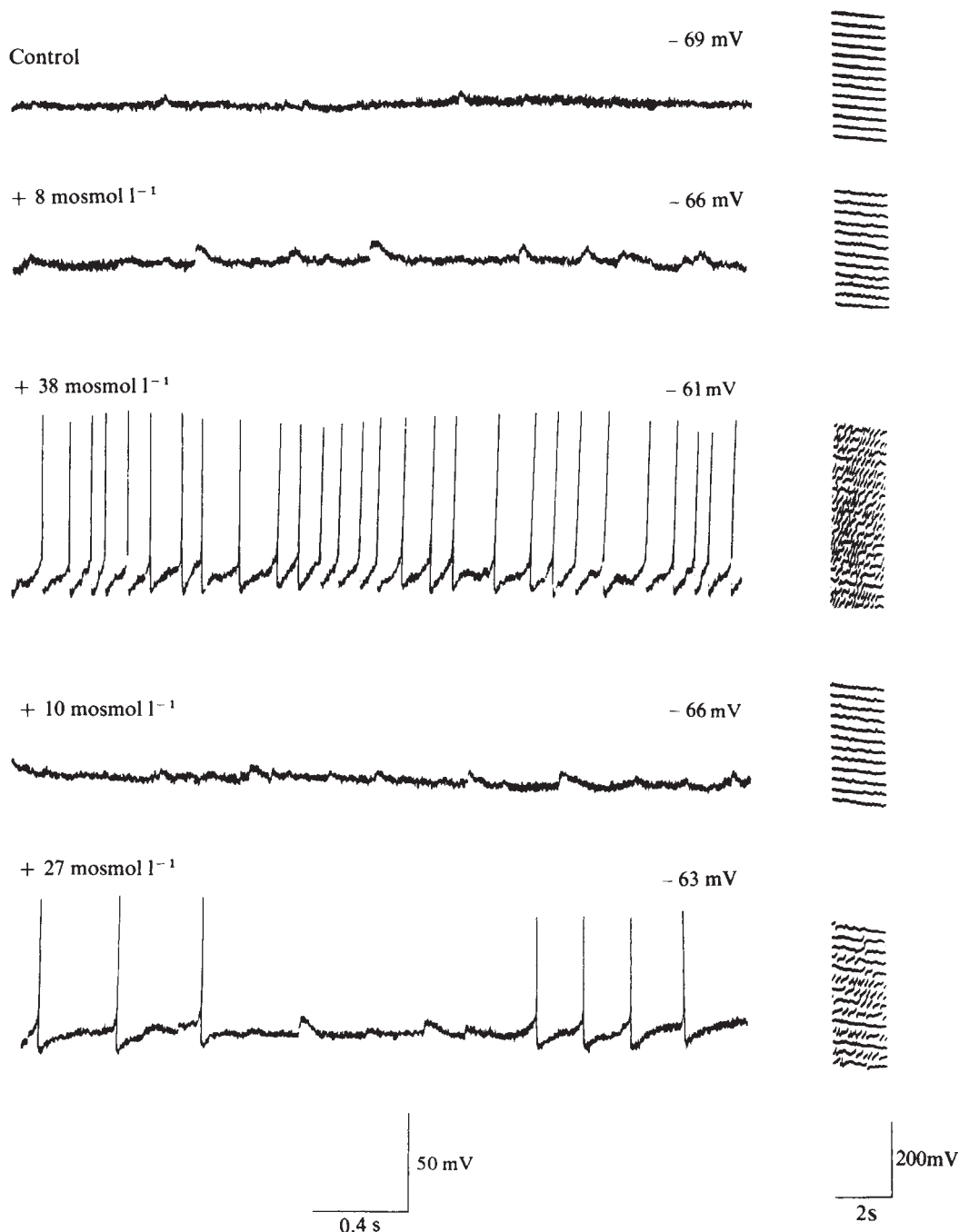


Fig. 1 Intracellularly recorded response from a neurone in the SON of the rat hypothalamus, recorded from a 400- μm slice maintained *in vitro*. Resting membrane potential (V_m) at the start of the record was -61 mV , and membrane resistance (R_m) measured with the bridge balance technique was $140\text{ M}\Omega$. The perfusing medium was changed where indicated to one of higher osmolarity by addition of mannitol, although similar results were obtained with NaCl in other experiments (as in Fig. 2). This cell responded with a clear increase in firing rate, accompanied by a depolarization of V_m of about 12 mV .

Fig. 2 Osmotic response of a supraoptic neurone recorded intracellularly over a period of 80 min ($R_m \sim 210 \text{ M}\Omega$). In these records, the time scale has been expanded to show the presence of baseline synaptic activity. On the top left of each record is given the presence of baseline synaptic activity. On the top left of each record is given the measured osmolarity change, elicited for this cell by addition of NaCl, with the control value before the start of the osmolarity change being 298 mosmol per kg. The baseline membrane potential for each cell is given on the top right. The records in the main body of the figure were reproduced by a Datalab transient recorder, and spikes have been retouched to represent their accurate height, which had been attenuated by the recorder. Records to the right of the figure are continuous traces photographed from the oscilloscope to show the longer-term baseline spontaneous activity of the same cell, and necessarily, the spike tops in these records have been lost. This cell has been illustrated to show the wide variation in activity which is elicited by osmolarity increase. While this cell would have been classed as a 'quiet' cell due to the lack of cell firing and low frequency of synaptic activity, it is apparent that a small and physiological osmolarity increase modifies both of these properties in a graded manner. Likewise, for a cell exhibiting spontaneous activity when impaled, osmolarity increase amplifies both the frequency of synaptic input and the firing rate of the cell. For this cell, it was possible to effect several changes of the bathing medium, such that a response to osmolarity could be observed, and reversed by returning to the control medium, followed by return to a medium of raised osmolarity about 10 mosmol per kg less than the first test. Resultant spiking was graded with the increase in osmolarity, being 9.2 Hz in the +38 mosmol per kg test and 2.4 Hz in the +28 mosmol per kg test (averaged over 5 min). The response to osmolarity consisted of depolarization of membrane potential, increased firing rate, and an increased frequency of synaptic events. As can be further seen, the initial increase in e.p.s.p. frequency occurred without resultant spiking, action potentials being observed only when V_m allowed the e.p.s.ps to approach threshold, which for this cell was about -50 mV . I.p.s.ps were also observed, although not as frequently as e.p.s.ps in this cell.



in 4–7 min. Osmolarity of the medium was measured with a Knauer semimicro-osmometer. Standard intracellular recording techniques were used, using electrodes filled either with 4M K acetate or 2M Na methylsulphate (resistance 70–250 $\text{M}\Omega$). Cells were occasionally marked intracellularly with the fluorescent dye Lucifer CH and examined in wholemount to confirm the recording site as being the supraoptic nucleus.

Some of the basic properties of intracellularly recorded neurones from this preparation have been described previously¹³. SON neurones recorded in slices of hypothalamus showed spike patterning similar to that observed *in vivo*^{8,15}, and, contrary to earlier reports on hypothalamic slices^{10–12}, considerable spontaneous activity. In isotonic medium, both phasic and continuous patterns of firing were noted, characteristic of vasopressin- and oxytocin-secreting cells, respectively⁸.

When the isotonic perfusion medium (290–295 mosmol per kg) was changed to one of increased osmolarity (305–340 mosmol per kg, with NaCl or mannitol), 19 of 23 cells responded with an overall increase in firing rate (Fig. 1). The increase in spike frequency was superimposed on a slow depolarization of membrane potential (V_m) ($13 \pm 3 \text{ mV}$ (mean $\pm$ s.d.) for a 35 mosmol per kg increase) graded with osmolarity over a range of 9–45 mosmol per kg. Of the cells responding in this way, three distinct response patterns were observed: (1) 'silent' cells ($<0.5 \text{ Hz}$) began to fire (1–10 Hz); (2) slow continuously firing cells (0.5–2.0 Hz) fired more rapidly, firing rate being graded with increase in osmolarity; (3) 'silent' cells and some phasic firing cells began to fire phasically and to fire with an increased burst length, respectively, some eventually firing in a continuous pattern.

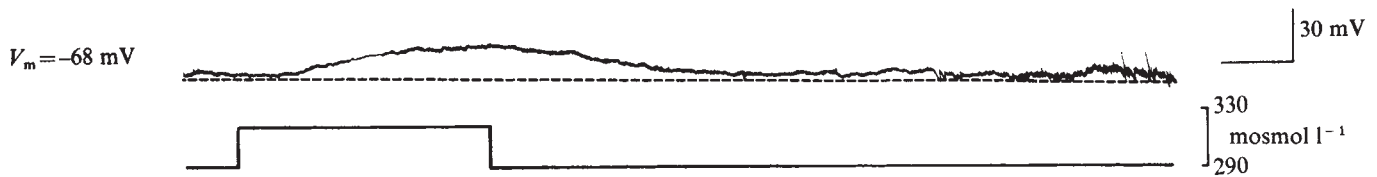


Fig. 3 The effect of increased osmolarity on a supraoptic neurone which had been blocked by increasing the Mg^{2+} content of the bathing medium. R_m was 165 M Ω , measured at the start of the record. Before the beginning of the experiment, after the cell had been impaled, 15 mM $MgSO_4$ was added and an equivalent amount of NaCl removed from the normal medium to maintain the isotonicity at 294 mosmol per kg. This treatment had no discernible effect on V_m . Following this, NaCl was added to raise the osmolarity as shown, and this resulted in a pronounced depolarization of V_m , but without a concomitant increase in firing rate as in Figs 1 and 2. Similarly, as synaptic input to the cell had been largely abolished by Mg^{2+} addition, synaptic potentials were not observed before, during or after increased osmolarity. However, the cell could be driven to fire by intracellular passage of depolarizing current such that V_m was brought above threshold (suprathreshold stimulation for this cell in the presence of Mg^{2+} elicited firing at about 50 Hz). Similar results were obtained where Mg^{2+} was added without removing NaCl, that is, where osmolarity was increased before a test by Mg^{2+} addition. This result indicates that the V_m depolarization resulting from increased osmolarity is a direct action on the primary neurone within the supraoptic nucleus, and not synaptically mediated or requiring synaptic input to the cell.

The firing patterns and depolarization of membrane potential induced by increased osmolarity were completely reversible when cells were restored to isotonic medium. Four 'silent' cells which did not respond to osmotic stimulus with increased firing did, however, depolarize in a manner comparable to responsive cells, but this depolarization did not, at the stimulus used, carry V_m sufficiently close to threshold for activation of firing. Current injected intracellularly through the recording electrode sufficient to depolarize the 'silent' cells beyond threshold elicited rapid firing, as observed in all cells.

During and preceding osmotically induced firing of SON neurones, significant changes in baseline voltage noise were observed. The magnitude of these changes varied from cell to cell and was graded with the measured change in osmolarity. The origin of this noise was a large increase in frequency of synaptic input to the cell, a property previously observed for more peripheral neurones¹⁶⁻²¹. This was analysed to study the correlation between membrane potential, cell firing rate, synaptic events and external osmolarity. Figure 2 shows that increased osmolarity has multiple and synergistic actions on both pre- and postsynaptic properties of SON neurones, in turn leading to an accelerated firing rate. These are increased synaptic input [excitatory postsynaptic potentials (e.p.s.ps) and inhibitory postsynaptic potentials (i.p.s.ps)], depolarization of the postsynaptic cell, and spike generation. For one typical cell, an osmolarity increase of 32 mosmol l^{-1} (w/NaCl) elicited (1) a peak depolarization of 14 mV; (2) increase in frequency of synaptic events (measured postsynaptically) from 3 s^{-1} to 34 s^{-1} (averaged over 5 min) and (3) increase in spike frequency from 1.4 Hz to 6.8 Hz. This observation of increased synaptic input to SON neurones, associated with a change in external osmotic pressure, raised the question of whether osmotically induced depolarization of membrane potential may also have been mediated by slower acting synaptic events, perhaps distant to the SON nucleus.

As demonstrated in Fig. 3, four SON neurones were tested for response to osmolarity increase (three with NaCl, one with mannitol) when they were bathed in 15 mM $MgSO_4$ solutions such that synaptic input was abolished. In three of these experiments $MgSO_4$ was added without removing NaCl and in a fourth experiment $MgSO_4$ replaced NaCl isotonicity. The osmotic stimulus was then applied and in all cells a substantial depolarization of V_m resulted, but in the absence of e.p.s.ps/i.p.s.ps and without firing. All cells continued to respond to injected depolarizing current with firing. These results were thus consistent with osmotically induced depolarization being a property of the primary neurone itself rather than a secondary, synaptically mediated event originating either within or outside the SON region.

Finally, the source of the presynaptic input to osmosensitive SON neurones remains a central question as these elements appear to be characterized by osmosensitive properties in their own right. To clarify this question, islands of SON were made from hypothalamic slices previously used for recordings. Not

only did nine intracellularly recorded neurones display spontaneous firing and synaptic input as in the intact slice, but also both of two cells tested showed an osmotic response similar to that described above. These observations, coupled with the similarity of response of neurones in coronal and sagittal sections, suggest that both the osmosensitive neurones themselves and the synaptic elements driving these cells lie near to and probably within the SON. The experiments do not of course rule out the possibility that more complex afferent pathways may exist *in vivo*. Inasmuch as the rat SON may not contain interneurones, these results could suggest a functional interaction between excitable SON neurones. In the light of reports that two-thirds of the synaptic input to SON neurones is of intranuclear origin, this might not be surprising²².

These findings conflict with earlier reports of extracellular recording from hypothalamic slice preparations, in that significant spontaneous activity as well as firing in response to osmolarity changes of the external medium is observed in cells recorded from the SON region. Many features of the spontaneous activity and osmotic responses in these intracellular records closely parallel those observed *in vivo* by means of extracellular recording from anaesthetized animals. These results must therefore strongly support the tentative suggestions of Jewell and Verney² as to the siting of the osmoreceptor in the anterior hypothalamus, in confirming that oxytocin- and vasopressin-secreting neurones lying within the SON are part of an excitable osmoreceptive complex. Furthermore, that the osmoreceptive complex as a whole (the pre- and postsynaptic element) lies within or near the SON is indicated by these results, although it cannot be excluded that the osmosensitive presynaptic element is a cut nerve ending of a more distant perikaryon. Whether the basic neuronal properties thus far observed for these osmosensitive cells distinguish them from other central neurones is open to speculation and will require a wide-ranging comparison with cells from other brain regions, but the siting of SON neurones within a dense capillary bed must make them well placed to detect and respond quickly and efficiently to changes in the osmolarity of the blood.

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Intracellular study of ionic events underlying intestinal membrane transport of oligopeptides

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It is now clear that after a protein meal much of the α -amino nitrogen which is absorbed from the small intestinal tract is transported into the intestinal epithelium across the brush border membrane of the enterocyte in the form of oligopeptides rather than free amino acids^{1–3}. We report here the results of electrophysiological experiments using intracellular microelectrodes which allow us to add important information concerning the mechanism of membrane transport which must underlie oligopeptide absorption, in particular with respect to the role of Na^+ in this peptide transport. Such experiments not only reveal the energetics involved in the major pathway for protein absorption from the gastrointestinal tract but may also be important for understanding the mechanism of action of small peptides in quite different cells, such as the Na^+ -sensitive binding of the neurohormone oligopeptide enkephalin to the enkephalin receptors in the central nervous system^{4,5}.

Figure 1 shows the results of a representative experiment in which the potential, V_m , across the brush border membrane of the superfused small intestine of the mudpuppy, *Necturus maculosus*, was recorded by a microelectrode inserted from the mucosal aspect into an epithelial cell⁶. The initial value of V_m (–45 mV) is somewhat greater than the mean value we find of 34 ± 2 mV (mean $\pm$ s.e.m., $n = 36$), which is almost identical to the mean value found in the same tissue by Garcia-Diaz *et al.*⁷. The cell was exposed initially to the amino acid L-leucine (6 mM), which produced a prompt, marked depolarization of 14 mV which was reversed completely on removal of the amino acid. This confirms previous findings^{8,9} and strongly suggests an ion-linked co-transport mechanism for entry of this neutral amino acid across the brush border membrane. Subsequent exposure to a higher L-leucine concentration (12 mM) produced a similar reversible depolarization which was almost identical to that found with the lower amino acid concentration, suggesting saturation of the brush border amino acid transport system. The cell was then superfused with a mixture of amino acid (12 mM) and the dipeptide L-leucyl-L-leucine (6 mM). This mixture produced a greater reversible depolarization; also, the rate of depolarization was increased by the presence of the dipeptide in addition to the saturating quantity of monomer. This increase must have been caused by the intact dipeptide rather than the products of extracellular or membrane digestion¹⁰ and therefore shows the existence of a pathway for peptide transport separate from and additional to that for the amino acid. Moreover, it shows that the entry of this neutral dipeptide into the cell via such a pathway is associated with an instantaneous flow of ionic current and thus that peptide transport is electrogenic.

Subsequently, the cell was exposed sequentially to 6 mM L-leucine, 12 mM L-leucine and the amino acid and dipeptide mixture (12 mM L-leucine, 6 mM L-leucyl-L-leucine); these solutes were then removed in reverse order. Exposure to the dipeptide produced a prompt 16 mV depolarization of the brush border membrane that was reversed on removal of the solute and was additional to that produced by exposure to a maximal saturating concentration of amino acid.

Ion replacement studies were carried out to elucidate the nature of the current flow induced by the dipeptide, and Fig. 2 shows the result of an experiment in which the effects of the amino acid and of the peptide on V_m were studied in a solution in which Na^+ had been totally replaced by choline. The measured Na^+ concentration in this solution after flow through the bath was 2 ± 1 mM (mean $\pm$ s.e.m., 6 observations). In this low concentration of Na^+ the effects on V_m of both amino acid and peptide were not totally abolished; however, the amino acid-induced depolarization was much slower than that seen in the presence of a high external Na^+ concentration (Fig. 1). With the dipeptide there is also a reduction in the rate of depolarization in low compared with high external Na^+ , although this reduction is considerably less for the dipeptide than for the amino acid.

These findings may help to resolve the controversy concerning the role of Na^+ in peptide transport. Matthews, Addison and Burston¹¹ showed that external Na^+ was required for the active accumulation of the poorly hydrolysed dipeptide L-carnosine (β -alanine-L-histidine) by rings of hamster intestine *in vitro*. Cheeseman and Parsons^{12,13}, however, found that the trans-epithelial flux of the amino acids glycine and leucine which had entered the tissue as the dipeptides glycyl-L-leucine or L-leucyl-glycine (both of which are hydrolysed intracellularly) was independent of the presence or absence of Na^+ from the intestinal lumen: they suggested that “the non-accumulative entry of the peptides into the epithelium can occur independently of the presence of Na in the lumen”. The results shown here clearly demonstrate electrogenic peptide entry for the easily hydrolysed, and therefore non-accumulated, dipeptide L-leucyl-L-leucine. The differing sensitivities of the peptide and the amino acid transport systems to a substantial decrease in external Na^+ , shown in Fig. 2, is most simply explained on the basis of the driving forces involved, as for any Na^+ co-transport

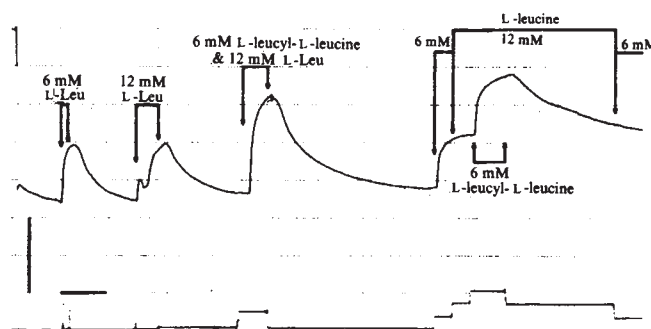


Fig. 1 Pen-recording of the potential across the brush border membrane of an intestinal epithelial cell of the mudpuppy *Necturus maculosus*. A piece of mid-duodenum was superfused at room temperature in a small Perspex chamber (vol. 0.8 ml) at a flow rate of 60 ml min⁻¹ with mudpuppy Ringer (Na 115, K 3, Ca 2.7, Cl 116, HCO₃ 2.4, PO₄ 3.0 mM l⁻¹; pH equilibrated with air, 7.4; osmolality 220 mosmol l⁻¹). At the start of the trace the cell was impaled from the mucosal aspect under direct visual control with a 24 MΩ glass KCl-filled microelectrode. When a stable recording had been established the composition of the superfusate was abruptly altered as shown (event marker on lower trace). The initial transient observed on exposure to 12 mM L-leucine is due to a small pulse of 6 mM H₂W7L-leucine preceding the higher concentration of amino acid. Vertical calibration 20 mV; horizontal calibration 60 s.

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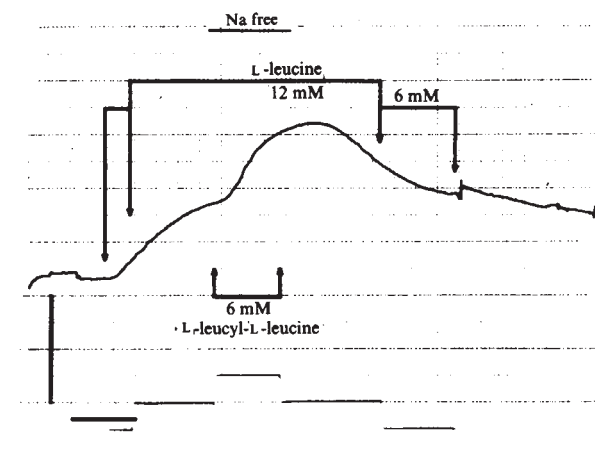


Fig. 2 Pen-recording of the brush border membrane potential of a small intestinal epithelial cell (see Fig. 1). The superfusate was identical to that used in Fig. 1 (except that Na^+ was totally replaced by choline) and its composition was altered as indicated (event marker on lower trace). Initial membrane potential was -47 mV. Vertical calibration, 20 mV; horizontal calibration, 60 s.

system the equilibrium potential¹⁴ is given by

$$V_{\text{equil.}} = \frac{RT}{F} \ln \frac{[\text{Na}^+]_o [\text{X}]_o}{[\text{Na}^+]_i [\text{X}]_i}$$

where $V_{\text{equil.}}$ is the reversal (equilibrium potential) for that co-transport and $[\text{Na}^+]_o$, $[\text{Na}^+]_i$, $[\text{X}]_o$ and $[\text{X}]_i$ are, respectively, the external and internal Na^+ and co-transported solute activities, and R , T and F have their usual meaning. Clearly, the magnitude of the depolarization observed on exposure to solute will be a function of both the driving force ($V_m - V_{\text{equil.}}$) and the passive membrane resistance¹⁵. For a readily hydrolysed peptide such as L-leucyl-L-leucine, the intracellular dipeptide activity is kept low because of intracellular peptidase activity; hence, the driving force for Na^+ -peptide co-transport will remain high even in solutions with low external Na^+ activity, and peptide transfer coupled to a flow of Na^+ will still occur at an appreciable rate. For both amino acids and poorly hydrolysed peptides in low external Na^+ , the driving force will be much reduced because of the intracellular activity of the co-transported substrate, and their uptake will therefore be strongly inhibited.

These experiments show the value of intracellular micro-electrode studies in defining the properties and mechanisms of a peptide transport system. By extending the approach to larger oligopeptides it should be possible to classify and map electrophysiologically the peptide transport systems of the brush border membrane. Moreover, the electrogenic nature of peptide transport revealed by such studies may be important with respect to both the evolution¹⁶ and role of small peptides as neurotransmitter and neurohormones in other tissues.

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Requirement of nerve growth factor for development of substance P-containing sensory neurones

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The protein nerve growth factor (NGF) is known to be essential for the maturation and maintenance of adrenergic neurones¹ and for the development of sensory neurones during critical stages of embryonic life²⁻⁴. The investigation of the physiological importance of NGF for the development of sensory neurones has been hampered so far by the lack of biochemical marker substances for these neurones. The demonstration that the undecapeptide substance P (SP) is present in sensory neurones^{5,6} suggests that it might be such a marker. SP is synthesized in dorsal root ganglia (DRG)⁷ and transported to the terminals of C-fibres located in the dorsal horn of the spinal cord and in the skin⁸. Its release can be demonstrated from the central^{9,10} and peripheral endings¹¹ of sensory nerve fibres which seem to have an important role in pain perception^{12,13}. We have investigated the effects of NGF and of purified anti-NGF antibodies on the content of SP in rat DRG and in their respective target organs, namely the spinal cord and the skin. The effects on sympathetic ganglia were included in order to control the effectiveness of both NGF and its antibody. We report here that NGF leads to an increase in SP in spinal ganglia, as previously shown by Kessler and Black¹⁴. However, in contrast to these authors, we describe that the administration of anti-NGF antibodies produces a marked reduction of the SP content in sensory neurones and in their respective nerve terminals.

2.5S NGF was prepared from submaxillary glands of adult male mice according to Bocchini and Angeletti¹⁵. Antibodies against 2.5S NGF were raised in goats and purified by affinity chromatography according to Stöckel *et al.*¹⁶. Two-day old Sprague-Dawley rats received subcutaneous (s.c.) injections of NGF (1 µg per g) during three consecutive days or a single s.c. injection of (50 µg per g) anti-NGF antibodies. Tyrosine hydroxylase (TH) activity was determined according to Mueller *et al.*¹⁷. Spinal ganglia (Th10-12), corresponding regions of spinal cord and pieces of skin from the hindlimb were prepared and homogenized in ice-cold 2 M acetic acid (2 ml). The samples were centrifuged, washed twice and the combined supernatants freeze dried. SP immunoreactivity was measured by a radioimmunoassay at a detection level of 6 pg SP according to Mroz *et al.*¹⁸. Protein concentrations were determined according to Lowry *et al.*¹⁹.

Repeated injections of NGF led to an increase in SP in rat spinal ganglia from 131 ± 20 to 200 ± 14 pg SP per ganglion (Fig. 1). The administration of anti-NGF antibodies caused a gradual decrease in SP up to 17 days, the longest time point studied (Fig. 1). This reduction was paralleled by a decrease in SP content of both the spinal cord and the skin (Table 1). The protein content of the spinal ganglia rose from 17 µg per ganglion at two days of age to 44 µg per ganglion at 19 days. Neither NGF nor its antibody had any significant influence on the protein content of DRG. The total TH activity in the superior cervical ganglion (SCG) showed a threefold increase after repeated administration of NGF, whereas the injection of

anti-NGF antibodies produced an almost complete disappearance of TH activity (Table 2), thus indicating optimal effects of NGF²² and of its antibody²³ on sympathetic ganglia.

The present experiments confirm that postnatal sensory ganglia are responsive to NGF and indicate that they require NGF or a cross-reacting NGF-like protein for their normal development, as evidenced by a decrease in their SP content after the administration of anti-NGF antibodies. Thus the concept that sensory neurones are dependent on NGF only during certain stages of their embryonic development² must be extended to the postnatal maturation of these neurones. Postnatal dependence may indicate a physiological function for the retrograde axonal transport of NGF found in sensory neurones^{24,25}. Future *in vivo* studies will have to show whether the modulation of SP by NGF is restricted only to defined stages of neuronal development. Our findings do not rule out the possibility that the reduction in SP effected by anti-NGF antibodies is due to neuronal death rather than to an impaired function of sensory neurones. The lack of a decrease in total protein content would, however, suggest that the anti-NGF antibodies only interfere with the functioning of DRG. Future studies will also have to investigate whether other neuropeptides found in sensory ganglia, like somatostatin and cholecystokinin²⁶, are affected by both NGF and its antibody. Previous studies have shown an effect of NGF on the SP content of DRG in explant cultures^{27,28}. Kessler and Black¹⁴ described a significant effect of NGF on the postnatal development of rat sensory neurones, while failing to find an effect of NGF antiserum. The discrepancy between these results and our findings may well be explained by the fact that the antiserum used was less potent than our purified anti-NGF antibodies, as evidenced by the decrease in TH activity in the SCG.

In conclusion, the present results indicate that NGF or a cross-reacting NGF-like molecule is important for the postnatal

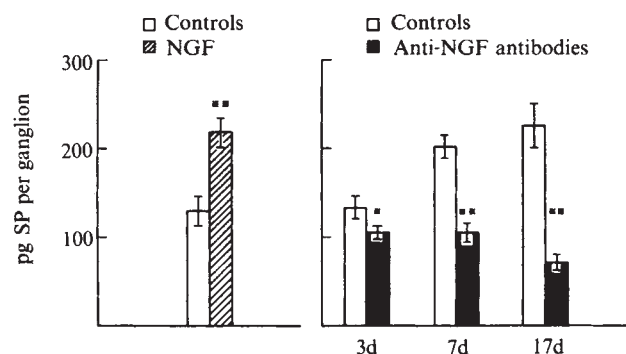


Fig. 1 Effects of NGF and anti-NGF antibodies on content of SP in rat DRG. Two-day old rats were injected s.c. with either $1 \mu\text{g g}^{-1}$ NGF or $50 \mu\text{g g}^{-1}$ anti-NGF antibodies. 2.5S NGF was prepared according to Bocchini and Angeletti¹⁵, with the modifications described by Suda *et al.*²⁰. The biological activity of NGF was determined according to Fenton²¹ and it amounted to 250 biological units per μg of protein. Antibodies against 2.5S NGF were raised in goats and purified by affinity chromatography¹⁶. Injection of NGF was performed on three consecutive days and SP content in DRG was examined one day after the last injection. Anti-NGF antibodies were injected once. Animals were killed 3, 7 and 17 d after injection and the DRG examined for SP content. Each value represents the mean $\pm$ s.e.m. ($n=4-6$ animals). * $P<0.01$; ** $P<0.005$.

development of SP-containing sensory neurones. They also suggest that, in addition to the drug capsaicin^{29,30}, anti-NGF antibodies might be a tool for reducing the SP content of primary sensory neurones.

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Table 1 Effects of anti-NGF antibodies on SP content of spinal cord and skin

Spinal cord	
Controls	206 $\pm$ 6
Anti-NGF antibodies	126 $\pm$ 13*
Skin	
Controls	28 $\pm$ 3
Anti-NGF antibodies	15 $\pm$ 2*

Two-day old rats were injected with $50 \mu\text{g g}^{-1}$ anti-NGF antibodies. They were killed 17 d later and SP content (ng per g of tissue) determined. Each value represents the mean $\pm$ s.e.m. ($n=5$ animals).

* $P<0.01$.

Table 2 Effects of NGF and anti-NGF antibodies (NGF-AB) on TH activity in the rat superior cervical ganglion (SCG)

	Controls	Treated
NGF	134 $\pm$ 12.4	388 $\pm$ 62.8*
NGF-AB 3 d after injection	122 $\pm$ 14.2	32 $\pm$ 1.3*
NGF-AB 7 d after injection	178 $\pm$ 14.2	21 $\pm$ 0.6*
NGF-AB 17 d after injection	242 $\pm$ 18.2	11 $\pm$ 0.2*

Two-day old animals were treated with either $1 \mu\text{g g}^{-1}$ NGF or $50 \mu\text{g g}^{-1}$ anti-NGF antibodies. NGF was injected on three consecutive days and the animals killed one day after the last injection. Anti-NGF antibodies were injected once and the animals killed 3, 7 and 17 d later. The SCGs were rapidly removed and homogenized in 5 mM Tris-HCl buffer, pH 7.4 (0.5 ml), containing 0.1% Triton X-100. TH activity was determined according to Mueller *et al.*¹⁷. Each value represents the mean $\pm$ s.e.m. ($n=4-6$ animals). Results are expressed as pmol dopa per h per pair ganglia.

* $P<0.005$.

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Tranquillizers can block mitogenesis in 3T3 cells and induce differentiation in Friend cells

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Compounds of diverse structure induce murine Friend erythroleukaemia (MEL) cell¹ differentiation²⁻⁵. Seeking a common property⁶, Bernstein⁷ reported that the relationship between activity and octanol/water partition coefficients for inducers resembled that reported for anaesthetics⁷; moreover, anaesthetics inhibited induction. Since anaesthetics were known to increase membrane fluidity, they suggested that inducers might decrease it. Reporting evidence against unitary theories of anaesthesia⁹, Richards *et al.*^{10,11} suggested that lipophilic drugs competed, according to their individual structure, with membrane lipids for hydrophobic regions on membrane proteins. The antagonism between pairs of anaesthetics^{10,11}, anaesthetics and inducers^{7,8} and pairs of inducers⁵, might thus be explained economically by Richards' anaesthesia model, inducers and lipophilic drugs acting by similar rather than contrary mechanisms. Lipophilic drugs should, therefore, induce differentiation. We report here that some tranquillizers do so. As reported elsewhere for classical inducers¹², they also block non-differentiating 3T3 cells in pre-S. These findings may have application in cancer chemotherapy.

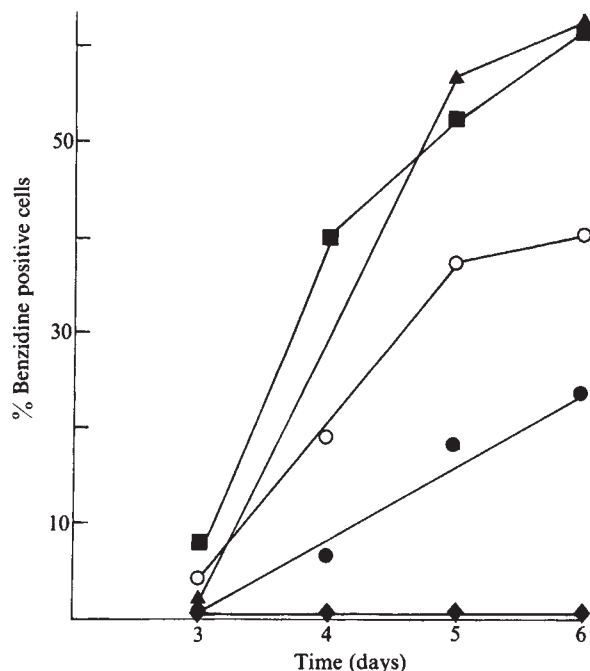


Fig. 1 MEL cells were seeded (as in Table 1 legend) at $3 \times 10^4 \text{ ml}^{-1}$ at 0 (◆), 8 (●), 16 (○) and $32 \mu\text{g ml}^{-1}$ (■) diazepam (dissolved directly into media) and 2.5% dimethyl sulfoxide (▲). Cultures were incubated at 37°C and duplicates removed daily and stained (Table 1 legend).

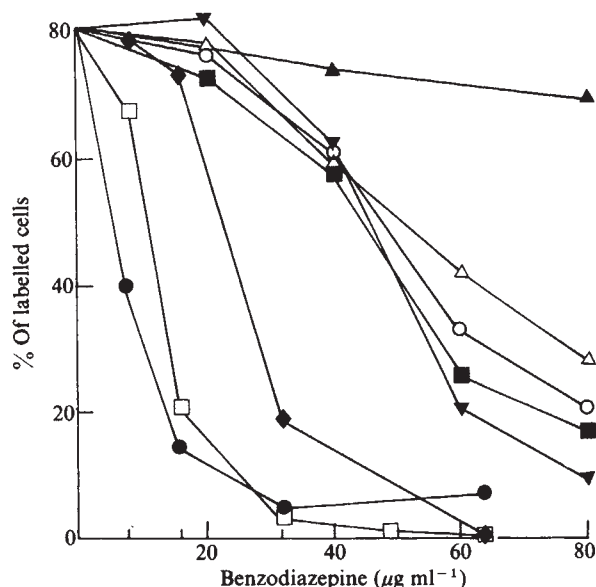


Fig. 2 Swiss 3T3 (4A reclone²⁷) cells were maintained in Dulbecco Eagle's medium containing 10% calf serum. Cultures with 2.5×10^4 cells in 2 ml of medium containing 0.5% serum were incubated in 3 cm plastic Petri dishes (Nunc) for 3 days at 37°C . Medium was then replaced with 6% serum medium containing $30 \mu\text{M}$ inosine, $2 \mu\text{Ci ml}^{-1}$ of tritiated thymidine (Radiochemical Centre, 21 Ci mmol^{-1}) and indicated concentrations of benzodiazepines: ■, chlordiazepoxide from 20 mg ml^{-1} in normal saline; ●, diazepam; ○, flurazepam; ◆, lorazepam; □, medazepam; △, nitrazepam; ▼, oxazepam; and ▲, temazepam, each from 100 mg ml^{-1} solutions in dimethyl sulfoxide. After 26 h incubation, cultures were fixed in formol saline and prepared for autoradiography¹⁶. The percentage of labelled cells was calculated from differential counts of 300 cells each on duplicate dishes. Cultures with 0.02–0.08% dimethyl sulfoxide did not differ from controls.

The benzodiazepines are a group of tranquillizers which induce anaesthesia in man on intravenous injection¹³, yet produce few toxic symptoms when high doses are administered orally or intraperitoneally to mice. We incubated MEL cells for 5 days with concentrations of eight benzodiazepines and stained with benzidine reagent¹⁴ to determine the proportion of the cell population which had accumulated haemoglobin (B+ benzidine positive). At up to lethal concentrations, three of the drugs induced differentiation (Table 1). Diazepam (Valium) and temazepam (Euhypnos) were highly active, giving rise to > 50% B+ cells at $40 \mu\text{g ml}^{-1}$. Figure 1 shows a time course indicating that $32 \mu\text{g ml}^{-1}$ of diazepam was as active as 2.5% dimethyl sulfoxide.

We report elsewhere¹² that a wide range of known inducers (dimethyl sulfoxide, hexamethylene bisacetamide, butyrate, ouabain and hypoxanthine) also inhibit the mitogenic effects of serum on 3T3 cells at inducing concentrations. Moreover, volatile anaesthetics such as halothane and nitrous oxide are known to inhibit cell proliferation¹⁵. We therefore tested the eight benzodiazepines for similar activity.

When 3T3 cells are incubated in medium with 0.5% serum they become quiescent with a low thymidine labelling index¹⁶. Incubation in medium with 6% serum for 26 h raised this index to 88% and this was reduced to below 35% by $16\text{--}80 \mu\text{g ml}^{-1}$ of seven of the benzodiazepines (Fig. 2). Diazepam was the most active but temazepam, an active inducer at $10 \mu\text{g ml}^{-1}$, had little inhibitory effect at $80 \mu\text{g ml}^{-1}$. The reduction in the 26-h labelling index was not the result of a brief delay in entry into S phase. In a 5-day growth experiment (Fig. 3), control cultures increased 18-fold but no proliferation occurred in diazepam ($64 \mu\text{g ml}^{-1}$). Time lapse cinematography showed that only 15% of cells

Table 1 Percentage of benzidine positive (B⁺) cells induced by benzodiazepines in MEL cell population

Drug	Drug concentration ($\mu\text{g ml}^{-1}$)			
	10	20	40	80
Chlordiazepoxide	<0.3	<0.3	3.4	D
Diazepam	18.5	37.4	52.9	D
Flurazepam	<0.3	<0.3	<0.3	D
Lorazepam	<0.3	<0.3	D	D
Medazepam	<0.3	<0.3	D	D
Nitrazepam	<0.3	<0.3	7.2	D
Oxazepam	<0.3	<0.3	0.7	D
Temazepam	24.1	ND	54.2	ND
None	2.7			

Cells of MEL subline F4²⁶ were maintained in equal parts of Dulbecco Eagle's and Hams F12 medium containing 7.5% fetal calf and 5% calf serum. Duplicate 1-ml volumes of medium, with no addition or with benzodiazepines, were seeded with 3×10^4 cells each in multidishes (Linbro). Chlordiazepoxide was dissolved initially to 20 mg ml^{-1} in normal saline, the other drugs to 100 mg ml^{-1} in dimethyl sulphoxide. After incubation at 37°C for 5 days, 200- μl aliquots were stained with benzidine reagent¹⁴ and the percentage B⁺ (blue) calculated from counts in a haemocytometer. Cultures with dimethyl sulphoxide at 0.02–0.08% did not differ from controls. Diazepam and temazepam dissolved directly into medium were equally active. D, Dead; ND, not determined.

divided and 10% died. When cells were changed to control medium after 48 h in diazepam, 99% divided in 31 h and an 18-fold increase occurred in 5 days (Fig. 3). Thus, diazepam is cytostatic rather than cytotoxic at this concentration.

Induction of differentiation by two tranquillizers supports the view that lipophilic drugs and inducers are a single group, active

by virtue of membrane solubility. Our finding that five known inducers¹² and now diazepam, inhibit 3T3 cells at concentrations at which they induce differentiation in Friend cells, is compatible with a hypothetical inverse relationship between the probabilities of proliferation¹⁷ and terminal differentiation^{18,19}. This relationship is also suggested by the differentiation in medium with reduced growth potential of normal muscle¹⁹ and adipose²⁰ cells. We have recently found that 5 of 16 further benzodiazepine derivatives tested induce differentiation in Friend cells. There is no correlation between this activity of the total of seven such inducers and their reported degree of inhibition of ³H-diazepam binding to rat brain membranes²⁸.

These findings may have applications in cancer chemotherapy. Agents which hold normal, but not malignant, cells in pre-S have been shown *in vitro* to protect them preferentially against S phase-specific anticancer drugs^{21–23}. Benign lipophilic drugs like Valium could conceivably do so *in vivo* (Fig. 3), thus increasing the therapeutic index of such drugs as antifolates, cytosine arabinoside, hydroxyurea and vinca alkaloids. They might also increase differentiation in tumours (see Table 1). Preisler *et al.*²⁴ have reported that an inducer of Friend cell differentiation may, in some situations, reduce tumour growth rate in mice. Many existing anticancer drugs are lipophilic and it seems possible that their action may not entirely conform with the antimetabolic roles for which they were originally tested. Screening of lipophilic CNS drugs used in man in tumour-bearing mice alone and as protective agents with other anticancer drugs could be worthwhile.

Gifts of drugs, diazepam, chlordiazepoxide, flurazepam, nitrazepam and medazepam (Roche), lorazepam and oxazepam (Wyeth), and temazepam (Montedison) are acknowledged. Dr Peter Riddle carried out the microcinematography and its analysis and Phil Whitehead assisted in some experiments. We thank Drs Robert Bürk and Ugo Rovigatti for helpful advice and discussion.

Note added in proof: A recent paper by Fehér and Gidali³⁰ describes the protective effect, for cycling cells of bone marrow against hydroxyurea, of actinomycin D, a lipophilic inducer of Friend cell differentiation³⁰. This paper has also drawn our attention to earlier reports of similar specific blocking of normal cell *in vivo* by halothane and nitrous oxide^{31–33}.

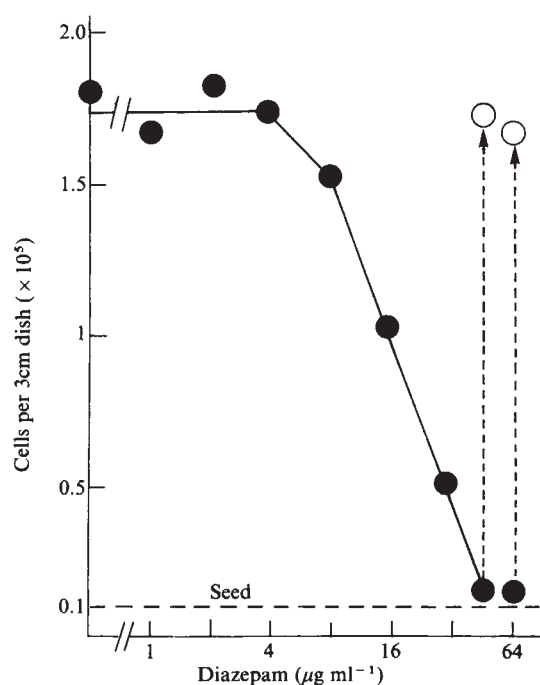


Fig. 3 Control cultures and those containing indicated concentrations of diazepam dissolved directly in 2 ml volumes of medium (Fig. 2 legend) containing 10^4 cells in 3-cm Petri dishes were incubated at 37°C for 5 days (●). Mean cell counts on duplicated dishes were made with an electronic Coulter counter. Duplicate cultures containing 48 and $64 \mu\text{g ml}^{-1}$ of diazepam were incubated for 48 h and medium then replaced with drug-free medium for a further 5 days incubation (○) before cell counts.

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Thymic lymphocytes bear a surface antigen which cross-reacts with acetylcholine receptor

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Acetylcholine receptor (AChR) is a major antigen in the neuromuscular disease myasthenia gravis and it is clear today that the basic defect in this disease is brought about by an autoimmune attack on acetylcholine receptors at the neuromuscular junctions^{1,2}. The involvement of the thymus and its role in myasthenia have been widely investigated but are still poorly understood. A high incidence of thymic abnormalities is observed in patients with myasthenia³ and thymectomy is beneficial in many cases⁴. Immunological studies have demonstrated the presence of humoral as well as cellular immune responses towards thymic tissues in myasthenic patients⁵⁻⁷. There were also some reports that animals immunized with thymic extracts develop a partial defect in neuromuscular transmission⁸. In spite of all these observations, the nature and origin of the association between the thymus and the neuromuscular junction in myasthenia gravis are still not known. We have previously demonstrated an immunological cross-reactivity, both humoral and cellular, between a thymic component and AChR⁹; such a cross-reactivity could provide a molecular explanation for the involvement of the thymus in myasthenia gravis. In this study, we demonstrate, by using immunofluorescence and radioimmunological techniques, that thymic lymphocytes bear a surface antigen which binds specifically to antibodies against nicotinic AChR and is thus defined as an 'AChR-like' antigen. A preliminary report of this study has been published¹⁰.

Thymuses from normal mice (C57BL/6 or SJL, 5-7 weeks old) were dispersed to cell suspensions in a phosphate-buffered saline containing 2% bovine serum albumin and 0.02% sodium azide (diluent). The cells were washed, centrifuges for 7 min at 1,200 r.p.m. and resuspended to a concentration of 10^7 - 10^8 cells ml⁻¹. Anti-AChR sera were obtained in various animal species by immunization with *Torpedo californica* purified AChR^{2,11}. For the immunofluorescence studies, 100 µl of the tested antiserum, immunoglobulin or purified antibodies were incubated for 30 min in the cold with 100 µl of thymic cell suspension (10^6 - 10^7 cells in the diluent) and the mixtures were centrifuged to remove the unbound proteins. The cells were washed twice and then reacted for an additional 30 min at 4 °C in the dark with the appropriate fluorescent reagent. The labelled washed cells were observed in the fluorescence microscope or were analysed in the fluorescence activated cell sorter (FACS)¹². For the radioimmunological measurements, the binding of anti-AChR antibodies to thymocytes was quantified by ¹²⁵I-protein A (Table 1)¹³.

As depicted in Fig. 1, thymocytes of normal C57BL/6 mice were specifically labelled by treatment with anti-*Torpedo* AChR immunoglobulins, followed by fluorescein- or rhodamine-conjugated goat anti-rabbit immunoglobulins. A large number of the reacted thymocytes (at least 80%) were fluorescently labelled (Fig. 1a, d). The fluorescence was evenly distributed on the cell surface in minute patches (Fig. 1a). Capping of the patches was observed following removal of sodium azide and further incubation at 37 °C (Fig. 1b). Immunoglobulins or serum from normal rabbit or anti-ovalbumin immunoglobulins did not result in any staining (Fig. 1c, f).

The microscopic findings were verified by quantitative analysis of the same samples in the FACS. Specific fluorescence peaks

were obtained following labelling with anti-AChR serum, immunoglobulins or purified antibodies (Figs 2 and 3). The labelled cells were shown to be the size of lymphocytes according to their light scattering. Increasing the amounts of the specific rabbit immunoglobulins added resulted in a shift of the peak towards higher relative fluorescence intensity (Fig. 2). It should be noted that C57BL/6 thymocytes were fluorescently labelled also with anti-*Torpedo* AChR sera prepared in monkey or even C57BL/6 mice (Fig. 3a, b). The reaction with mouse (C57BL/6) anti-AChR serum was of lower intensity than those obtained with rabbit or monkey sera and could be detected only in the higher sensitivity of the FACS (660 V, Fig. 3b). Specific binding of anti-AChR sera or immunoglobulins to mouse thymocytes was also observed by radioimmunoassay measurements (Table 1).

Several experiments were performed in order to verify the specificity of the binding to thymocytes. Thus, it was shown that purified anti-AChR antibodies (purified on an AChR-Sepharose adsorbent) bound specifically to thymocytes (Fig. 3c, Table 1). Absorption of anti-AChR antibodies on an AChR-Sepharose adsorbent¹⁴ abolished the ability to bind to thymocytes. These two experiments provide direct proof that the reacting antibodies are indeed anti-AChR antibodies and rule out the possibility that antibodies of other non-AChR specificities which could be present in the immunized animals, due to additional autoimmune responses, could react with the thymocytes. Moreover, immunoglobulins or purified antibodies from non-myasthenic rabbits immunized with denatured AChR

Table 1 Binding of anti-AChR to mouse thymocytes

Thymocytes	Sample	Binding (c.p.m.)		
		(dilution)		
	Sera	1/2	1/4	
C57BL/6	Rabbit anti-AChR	46,420	37,310	
	Rabbit anti-RCM-AChR	49,140	47,420	
	Rabbit anti-(T, G)-A-L	16,250	10,200	
	Rabbit normal serum	20,100	N.D.	
C57BL/6	Monkey anti-AChR	75,210	68,420	
	Monkey normal serum	20,210	19,460	
C57BL/6	Mouse (C57BL/6) anti-AChR	46,320	22,470	
	Mouse (C57BL/6) normal serum	19,200	9,720	
	Immunoglobulins	750	375	180
C57BL/6	Rabbit anti-AChR	51,250	35,720	12,910
	Rabbit anti-ovalbumin	7,870	9,000	8,840
	Purified antibodies	24	18	
C57BL/6	Rabbit anti-AChR	10,510	4,950	
	Rabbit anti-RCM-AChR	N.D.	4,610	
	Rabbit anti-ovalbumin	1,950	40	
SJL	Rabbit anti-AChR	8,720	2,920	
	Rabbit anti-ovalbumin	1,130	230	

Thymocytes (10^5 - 10^6 suspended in 50 µl of diluent) were added to aliquots, (100 µl) of the tested sera or immunoglobulins, in wells of microtitre plates. Following an incubation at 4 °C for 30 min, the plates were centrifuged and washed twice with the diluent. ¹²⁵I-protein A¹³ was added (100 µl per well, approximately 150,000 c.p.m.) and the plates were incubated in the cold for an additional 60 min. The plates were centrifuged and washed twice and the wells were separated and counted. Each dilution or immunoglobulin sample was assayed in triplicate and the amount of binding was determined after subtraction of the background radioactivity obtained by incubation of the thymocytes with the diluent instead of the tested serum or immunoglobulin. The background values were about 3,500 c.p.m. and 1,000 c.p.m., respectively, for the two different preparations of ¹²⁵I-protein A used for these experiments. The first preparation was utilized in the experiments with sera and immunoglobulins and the second one in the experiments with purified antibodies.

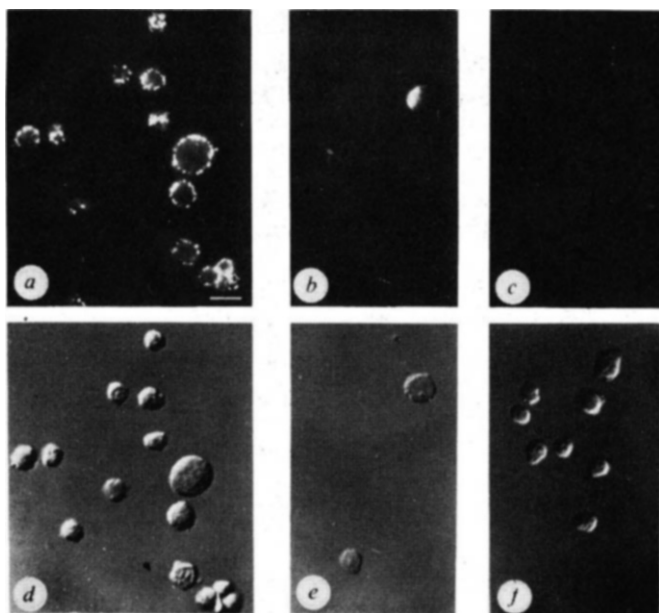


Fig. 1 Immunofluorescent staining of C57BL/6 thymocytes with anti-AChR antibodies. *a, b*, Epifluorescence of cells incubated with rabbit anti-AChR IgG followed by rhodamine-goat anti-rabbit IgG. *d, e*, Same fields photographed with Nomarski optics. Cells were incubated at 4 °C in the presence of 10 mM NaN₃ (*a, d*) and at 37 °C in the absence of azide (*b, c*). *c, f*, Control: cells were incubated with rabbit anti-ovalbumin IgG instead of anti-AChR. Other conditions are as in *a, d*, respectively. Scale bar, 10 μ m.

preparation (reduced carboxymethylated AChR, or RCM-AChR¹⁵) also bound to thymocytes (Fig. 3*d*, Table 1).

In all the experiments described so far, thymocytes from C57BL/6 mice were tested. This mouse strain was shown to be susceptible to experimental autoimmune myasthenia gravis (EAMG)¹⁶. Similar experiments were performed with thymocytes from a non-susceptible strain¹⁶ in order to test whether the non-susceptibility to EAMG in the latter mice could result from the absence of the 'AChR-like' antigen on their thymocytes. Experiments performed with thymocytes of SJL mice also demonstrated a specific binding of rabbit and monkey anti-AChR sera and of rabbit anti-AChR purified antibodies (Table 1), similar in intensity to that obtained with C57BL/6 thymocytes.

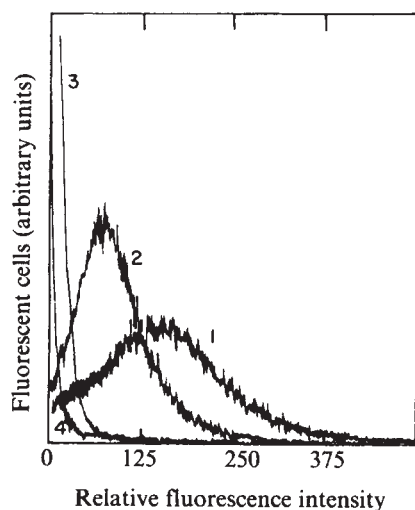


Fig. 2 Immunofluorescence profile of C57BL/6 thymocytes labelled with rabbit anti-AChR immunoglobulins (1, 2, 600 and 200 μ g, respectively) and with rabbit anti-ovalbumin (3, 4, 600 and 200 μ g, respectively). Fluorescein-conjugated goat anti-IgG was used for staining.

mocytes. These results indicate that the non-susceptibility of SJL mice to EAMG is not associated with a lack of the 'AChR-like' antigen in the thymus.

The results reported here suggest that the component in thymocytes which cross-reacts with anti-AChR antibodies is a surface antigen. The fluorescence studies also demonstrate that the 'AChR-like' antigen on thymic lymphocytes is homogeneously distributed on the cell surface and that a similar number of antibody molecules are bound per cell under a given antibody concentration. It seems that the amount of antibodies reacting with thymocytes in anti-AChR sera is probably rather low or, alternatively, of very low affinity because the cross-reactivity could be observed only by using relatively high amounts of anti-AChR specific sera. We are attempting now to isolate by specific immunoadsorption and to identify the AChR cross-reacting material from the thymus.

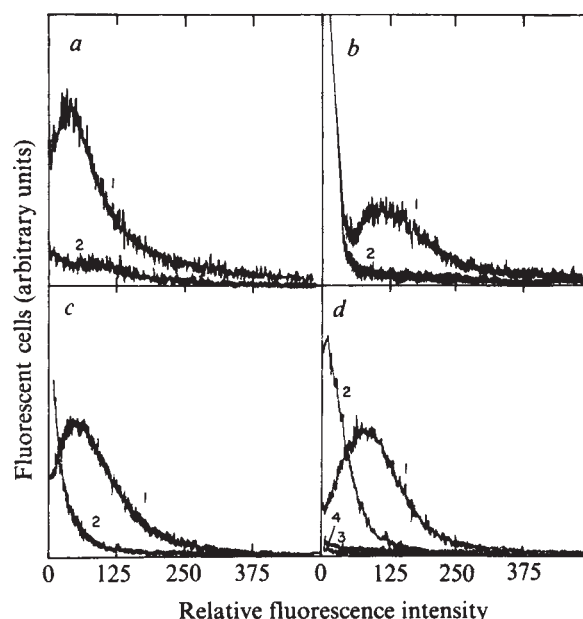


Fig. 3 Immunofluorescence profiles of C57BL/6 thymocytes. *a*, Labelling with monkey anti-AChR serum (1) and with monkey normal serum (2), followed by fluorescein-conjugated protein A (Pharmacia). *b*, Labelling with C57BL/6 mouse anti-AChR serum (1) and with C57BL/6 mouse normal serum (2) followed by fluorescein-conjugated goat anti-mouse IgG. *c*, Labelling with rabbit anti-AChR purified antibodies ([1], 30 μ g) and with rabbit anti-ovalbumin IgG ([2], 30 μ g) followed by fluorescein-conjugated goat anti-rabbit IgG. *d*, Labelling with rabbit anti-RCM-AChR serum (1, non-diluted; 2, 1:4 dilution) and with rabbit anti-ribonuclease serum (3, non-diluted, 4, 1:4 dilution) followed by fluorescein-conjugated goat anti-rabbit IgG.

It should be pointed out that we did not succeed in demonstrating any significant toxin-binding activity in thymocytes or thymic extracts. This is also in agreement with the finding that antibodies against a pharmacologically inactive RCM-AChR cross-react with thymus (Fig. 3*d*). These results suggest that the immunologically cross-reacting material on thymocytes is probably not an active nicotinic receptor. Nevertheless, an immunological cross-reactivity by itself could explain the association between the thymus and the neuromuscular junction in myasthenia, where the thymic antigen or a modified form of it could be the primary antigen in the spontaneous disease, or alternatively could be damaged by a cross-reactive autoimmune attack by anti-AChR antibodies. Previous reports have suggested that the immunological cross-reactivity between AChR and thymus could stem from myoid cells which were shown to develop to striated muscle fibres in cultures of thymus reticulum under selective medium conditions^{17,18}, or from some thymic

epithelial cells which were shown to contain acetylcholine receptor¹⁹.

The presence of anti-thymus antibodies has been demonstrated in a high percentage of myasthenic patients⁷ and we could also detect binding of sera from myasthenics to mouse thymocytes. However, in the spontaneous disease, there is no direct evidence whether the immune response to thymus is a separate independent reaction or whether autoantibodies to AChR cross-react with the thymus. The present study provides

direct evidence that anti-AChR antibodies can cross-react with the thymus.

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Epstein-Barr virus-induced cell fusion

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Serological and molecular biological studies¹⁻³ have shown an association between Epstein-Barr virus (EBV) and nasopharyngeal carcinoma. Although it has been shown that the epithelioid tumour cells carry EBV genomes⁴, they are apparently devoid of receptors for EBV (H.W., unpublished observations). Others have suggested that fusion of EBV carrying cells with epithelial cells may be the mode of entry of the virus into cells unable to absorb the virus and that this may be mediated by one of the known syncytium-forming viruses which inhabit the respiratory tract (for example, members of the paramyxovirus group). de Thé and colleagues suggested that intercellular bridges could be seen in NPC tumour material⁵. We have developed a technique which permits the preparation of stable monolayers of viable human lymphoblastoid cell lines⁶. Using this technique we have now demonstrated that EBV can induce fusion between EBV-superinfected lymphoblastoid cells and cells devoid of EBV receptors.

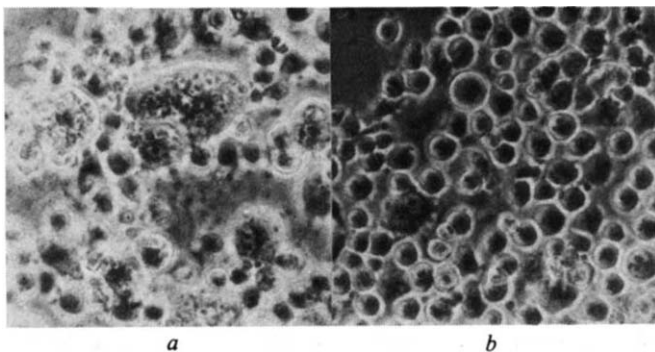


Fig. 1 The formation of polykaryocytes in cultures of immobilized superinfected Raji cells. A stock of EBV prepared from P3HR1 cells was used to superinfect Raji cells as previously described⁸. After shaking at room temperature for 1 h, the cells were applied to plastic Petri plates coated with ALG. The infected, immobilized cultures were incubated at 37°C. *a*, Raji cells superinfected with concentrated virus, 4 h post-infection. *b*, As *a*, but virus diluted 1:100. $\times 650$.

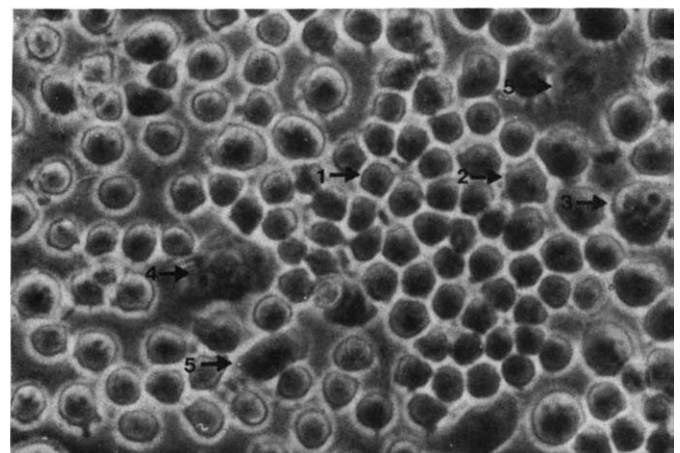


Fig. 2 The formation of polykaryocytes between superinfected and uninfected Raji cells. 10^6 Raji cells were shaken with 0.5 ml of an undiluted EBV stock at room temperature for 1 h. The cells were washed to remove unadsorbed virus and mixed with 9×10^6 Raji cells. The mixture was applied to ALG-coated plates as described in Fig. 1 legend. After 48 h of incubation at 37°C, the culture was photographed. The various stages of polykaryocyte formation are indicated by arrows: (1) single cells; (2) binucleate cells; (3) cells with up to 10 nuclei; (4) nuclei begin to fuse and disintegrate; and (5) total loss of nuclear identity and disintegration. $\times 1,400$

Raji cells, which carry the EBV genome but do not express early or late viral antigens, are susceptible to superinfection with EBV derived from the EBV-producing cell line P3HR1. Superinfected cells enter an abortive or productive cycle of virus replication depending on the multiplicity of infection. Productively infected cells synthesize viral DNA⁷ and several virus-specified polypeptides⁸ including early antigen (EA)⁹ and viral capsid antigen (VCA)¹⁰. This superinfection system was used as a model to study the interaction of EBV-infected B-lymphoblastoid cells with one another and with unrelated cells. The response of uninfected Raji cells to immobilization on plastic surfaces coated with antilymphocyte globulin (ALG) has been described elsewhere⁶. When superinfected Raji cells were applied to ALG-coated plates in sufficient numbers to yield a confluent monolayer, polykaryocytes were observed within 2 h of immobilization (4 h after addition of the virus to the cells). Figure 1*a* shows a phase contrast photomicrograph of such a culture. Dilution of the virus stock used to superinfect the Raji cells leads to a diminution of the number of polykaryocytes

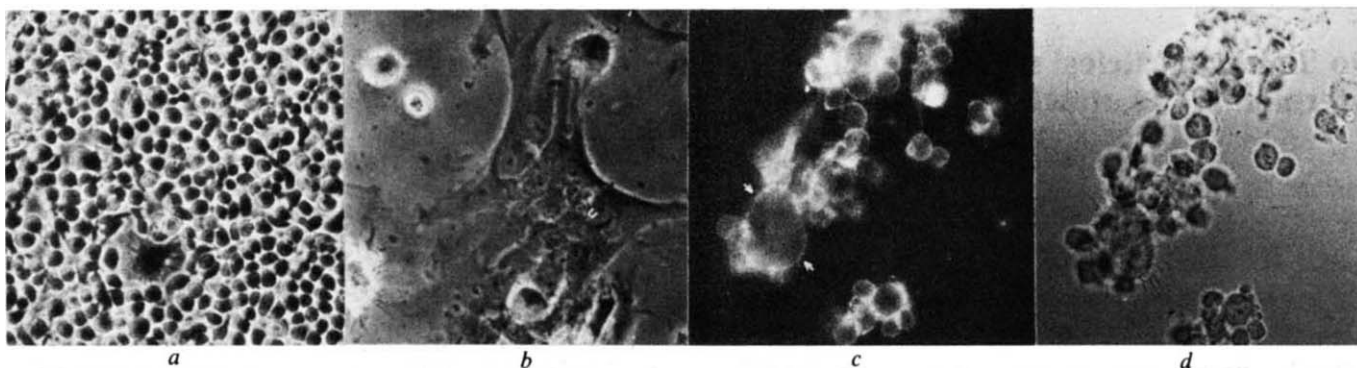


Fig. 3 The formation of polykaryocytes between infected Raji cells and cells devoid of EBV receptors. Raji cells were applied to ALG-coated plates to yield very sparse monolayers (approximately 10% confluent). After the cells had attached, virus was added and 1 h later either Jurkat (T-lymphoblastoid cells; *a*, *c*, *d*) or human embryo fibroblasts (*b*) were added to give a subconfluent monolayer. The cultures were incubated at 37 °C and photomicrographs prepared 20 h after infection. The cultures in *c* and *d* were stained with anti-T-cell serum. *c* Shows a polykaryocyte with T-cell membrane fluorescence. The arrows indicate areas where the polykaryocyte is free of adherent cells (see *d*) and shows clear membrane fluorescence. *d* Is a photograph of the same group of cells taken under normal light. At least three nuclei can be identified within the polykaryocyte. *a*, *b*, $\times 650$; *c*, *d*, $\times 1,000$.

observed (Fig. 1*b*; virus diluted 1:100). Immobilization of Raji cells without addition of exogenous EBV does not induce polykaryocyte formation. With minor modifications this technique can be used to titrate stocks of EBV. A similar series of experiments, in which infected cells were treated with sodium azide or cycloheximide, or in which cells were infected with UV-ray inactivated virus or virus treated with neutralizing antiserum, indicated that at least partial expression of EBV is required to induce cell fusion.

The high multiplicities of infection necessary to induce a full or partial virus reproductive cycle prevents observation of the interaction between infected and uninfected cells. To obviate this problem, superinfected Raji cells were washed to remove unadsorbed virus and mixed with uninfected Raji cells in a ratio of 1:10. The mixture of cells was applied to ALG-coated plates and the cells were allowed to attach as described above. After 48 h, numerous multinucleate cells could be observed and in some of the syncytia the nuclei had begun to disintegrate or coalesce. Figure 2 illustrates the various stages of syncytium formation.

These experiments show that EBV can induce fusion between cells having receptors for EBV, but not whether EBV can induce fusion between receptor-positive and -negative cells. We studied the interaction of superinfected Raji cells with human T-lymphoblastoid cells (Jurkat) or human embryo fibroblasts (HEF). Raji cells were applied to ALG-coated plates to give very sparse monolayers with a cell density of less than 10% of confluency. These cells were infected *in situ* by adding a stock of EBV; after the virus had been adsorbed by the Raji cells, Jurkat or HEF cells were added. After incubation at 37 °C for 20 h, the cultures were photographed (Fig. 3*a*, *b*). From these photographs it is apparent that the cells had fused giving rise to polykaryocytes. Fluorescent antibody staining of the cultures showed that the polykaryocytes contained EBV antigens (EA and VCA) and that the polykaryocytes formed between superinfected Raji cells and Jurkat cells had T-cell membrane determinants on their surface (Fig. 3*c*, *d*).

Our observations suggest that close cell-to-cell contact, as it occurs in closely packed monolayers, is necessary for the development of multinucleate cells. A close contact between epithelial cells and lymphocytes (which may carry EBV genomes) has been observed in the lymphoepithelium of Waldeyer's ring of the throat¹¹. The exceptionally close contact between the lymphocytes and epithelial cells in this unique tissue may provide the necessary conditions for EBV-induced fusion between the two cell types. For further considerations of

this aspect, and the relationship of EBV to other lympho-epithelial and undifferentiated carcinomas of the Waldeyer's ring see ref. 12.

In situ hybridization studies¹³ with NPC tumour material indicate that the cancerous cells carry a heavy load of viral genomes. This could be achieved in one of two ways: either the virus is activated, enters into a lytic cycle and replicates independently of the host cell using virus-specified DNA polymerase¹⁴ or, as in cell lines which carry the genome but cannot enter a lytic cycle, the viral DNA can be replicated using host replicative machinery (for example, see ref. 15). Lymphoblastoid cells in a lytic cycle which contain several thousand copies of the viral genome¹⁶ could fuse with epithelial cells transferring its entire viral load. Tumour cells contain large quantities of viral DNA, the near diploid or near triploid karyotype and chromosomal abnormalities¹⁷ fusion of EBV-carrying lymphocytes with epithelial cells could generate such abnormal cells. In addition the hybrid cell could then eliminate most of the genetic material originating from the lymphoblastoid cell and regain a near normal chromosome number. The efficiency with which EBV can induce cell fusion in tissue culture supports the hypothesis that EBV-induced cell fusion may be important in the infection of epithelial cells, which are normally refractory to infection by this virus, provided that certain conditions of virus activation and close cell contact are met.

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Do 'lipidic particles' represent intermembrane attachment sites?

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Although freeze-fracture electron microscopy is normally analysed with the presumption that particles represent proteins embedded within the membrane, particles which appeared to represent inverted lipid micelles within membranes have been reported¹⁻⁴. I have now confirmed the occurrence of particles in protein-free liposomes, but have found that they behave like intermembrane attachment sites rather than structures within a membrane.

Pits and particles have recently been described on freeze-fracture replicas of liposomes containing an equimolar ratio of cardiolipin and phosphatidylcholine¹. Furthermore, several lipids which are capable of forming a hexagonal II phase will also form pits and particles on freeze-fractured membranes^{2,3}. The interpretation of these results has been that these lipids are able to form inverted micelles, which have a similar organization to the hexagonal II phase, sandwiched between the leaflets of the membrane^{1,2,4}. It has also been suggested that some of the particles seen in freeze-fractured biological membranes may also represent inverted lipid micelles in the centre of the membrane^{1,2,4}. This is a serious suggestion since, thus far, freeze-fracture replicas have been analysed with the consideration that particles represent proteins (with or without a layer of adsorbed lipids and/or condensate) intercalated in the plane of the membrane. I have now studied a cardiolipin-phosphatidylcholine liposome system to define better the nature of the particles and pits in these membranes.

Multilamellar liposomes were prepared as follows: an ethanol solution of cardiolipin (Sigma) and phosphatidylcholine (egg yolk, Sigma) was dried under a stream of dry nitrogen and hydrated with buffer (100 mM NaCl, 10 mM Tris (pH 7.5) with 10% glycerol). The resulting suspension was vigorously shaken for 10 min and sonicated in a bath type sonicator for 10 min. Similar results to those presented have been obtained without glycerol in the buffer and by using the injection method of liposome preparation (see ref. 1 for example). Addition of calcium was performed by addition of an appropriate aliquot of the same buffer containing 10 mM CaCl₂ and mixing. Liposomes were quenched in partially solidified freon and freeze-fractured in a Balzers freeze-fracture device.

Multilamellar liposomes formed with an equimolar ratio of cardiolipin and phosphatidylcholine are spherical, smooth and do not contain particles or pits (Fig. 1a). The addition of calcium (2 mM final concentration) induces the appearance of particles and pits on some of the liposomes. In most of the liposomes which showed particles or pits, the fracture face exposed was that of the external membrane of the liposome, and contained either particles (Fig. 1b) or pits (Fig. 1c) but not a mixture of the two. In each case, the leaflet which contained the particles was the external leaflet (E-face) and the leaflet which contained the pits was the internal leaflet (P-face). No fractures which apparently represented the external membrane of a liposome contained pits on the E-face or particles on the P-face. Less than 5% of the liposomes were fractured such that internal membranes of the liposome were revealed. On those that were, I found fracture faces with either particles or pits, and on some occasions a mixture of both (Fig. 1d). Consistent with the report that calcium induces fusion in cardiolipin-containing liposomes³, many liposomes were found that appeared to be

partially fused. These will be the subject of a subsequent report from this laboratory (work in progress).

The location of the pits and particles on these membranes was not random: typically they appeared in linear arrays, the individual particles or pits being separated from each other by 12–40 nm. This indicates that long-range interactions are responsible for the location of the pits and particles. Linear arrays of particles occurred along the top of slight ridges on the fracture face, while linear arrays of pits occurred at the bottom of complementary valleys. This effect creates the quilt-like pattern seen in Fig. 1b–d. In preparations where the calcium concentration was increased to 6–10 mM, few liposomes remained intact and under the dissecting microscope, massive precipitation of the liposomes was observed. In replicas, the central region of the precipitate no longer appeared as individual bilayer membranes. However, at the edges of the precipitate, regions could be found which appeared to retain the bilayer configuration and which were characterized by high densities of either particles or pits (Fig. 1e).

Several observations must be reconciled in a hypothesis which attempts to explain the data: (1) the highly asymmetrical distribution of the particles and pits. In all cases, the outer membrane E-face contained particles while the P-face had pits; (2) the arrangement of the pits and particles into linear arrays; (3) the occurrence of the pits in valleys and particles on ridges of membrane contour; and (4) the absence of particles or pits on many membranes in the preparations. In Fig. 1g–j, I present several hypotheses which have been considered: in g the model of an inverted micelle in the centre of the membrane is symmetrical with respect to the membrane and cannot be expected to have long-range interactions to alter the contour of the membrane. In h, the membrane is distorted to produce a particle-pit pair which is asymmetric but does not have the potential for long-range interactions any further than the diameter of the pit and would not be expected to occur in valleys or ridges of membrane contour. In i, a variant of h, the production of a pit-particle pair is accompanied by a larger deformation of the membrane contour of a diameter, x . This model is asymmetrical, it could provide long-range interactions between the pits or particles up to a distance x (minimization of surface energy is expected to aggregate such particles), and the distribution of particles and pits on ridges and valleys, respectively, could be accounted for. That the particles do not appear on all regions of the membrane is not, however, accounted for. Finally, in j, the particles and pits are proposed to be attachment sites between two membranes. This model reconciles all the observed data: (1) Such attachments between two membranes necessitate the proximity of another membrane. An observed leaflet would demonstrate pits if the membrane to which it is attached is beneath the fracture plane, and particles if it is above the fracture plane. Both pits and particles would be seen only if the observed membrane is attached to membranes both above and below it. (2) If the attached points are mobile within the plane of the membranes, distension of the spaces between two such membranes can be expected to aggregate the points into a line or, in the extreme case, a cluster. (3) A row of points would be expected to alter the local contours of the membranes such that particles would appear at the tops of ridges and pits at the bottoms of valleys. This would produce the quilt-like pattern which is typical of these preparations; and (4) Although specific lipids (for example, cardiolipin in the presence of calcium) may be a necessary condition for the formation of these pits and particles, the ability to make contact (or maintain contact) with another membrane may be the limiting factor in the production of pits and particles.

Among morphologists, concern has arisen that many of the particles which have been presumed to be indicative of proteins may be purely lipidic. However, the present analysis suggests that lipidic particles may represent sites of membrane contact; this should greatly decrease occasions for concern in biological systems.

A more crucial question arising from the present analysis is

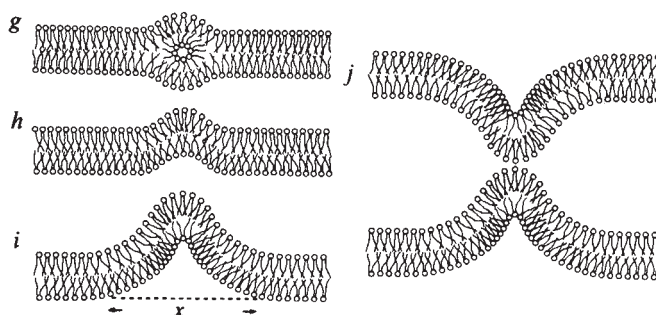
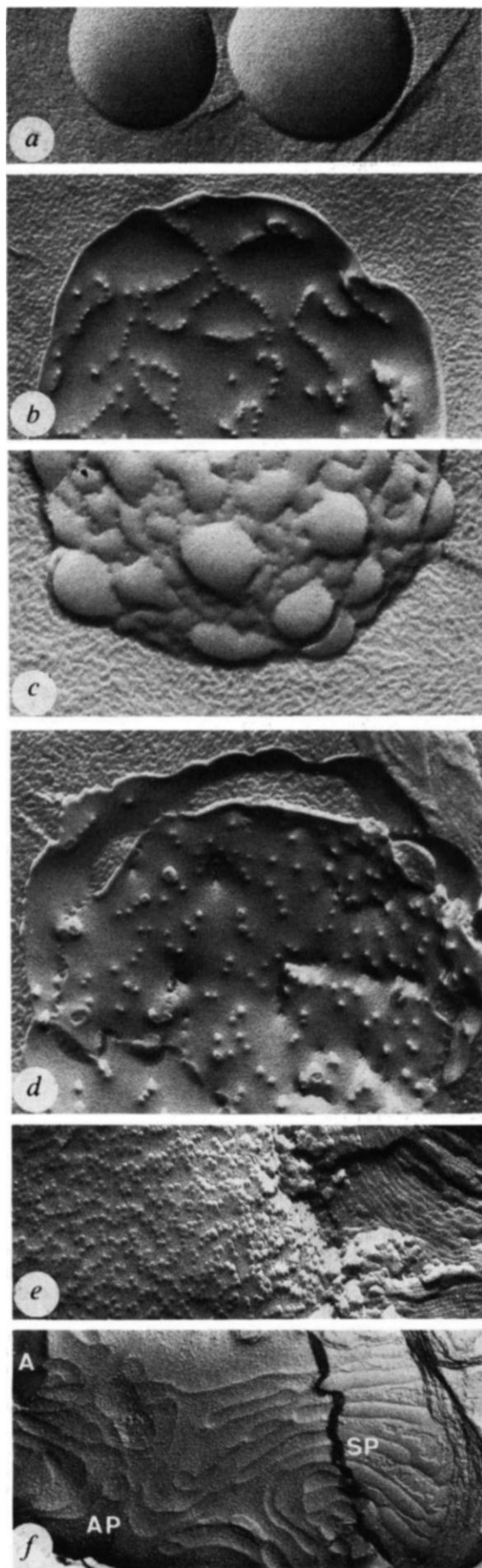


Fig. 1 *a*, Liposomes of 1:1 molar mixtures of cardiolipin:phosphatidylcholine before addition of calcium have membranes which are smooth and uninterrupted by pits or particles: $\times 80,000$. *b*, Addition of calcium to a final concentration of 2 mM induces the appearance of particles on the outer leaflet of the liposome: $\times 60,000$. From the same preparation as *b*: *c*, the inner membrane leaflet of the outer membranes of these liposomes show pits: $\times 80,000$. And *d*, occasionally fractures expose membranes which are not the outer leaflet of the liposome. In these cases, pits, particles or mixtures of the two (as shown here) are present: $\times 65,000$. *e*, When the concentration of calcium is increased to between 6 and 10 mM, precipitation of the liposomes is apparent. At least two phases are present, one with bilayer conformation and many pits or particles, and the other lamellae with no visible aqueous layer between the lamellae: $\times 80,000$. *f*, Freeze fracture of a rat sciatic nerve frozen immediately after removal from the animal. A, axoplasm; AP, axolemmal P-face; SP, Schwann cell P-face: $\times 25,000$. *g-j*, Several hypothetical models which may be considered to explain 'lipidic particles'. *g* and *h* are redrawn after ref. 2. See text for further details.

whether structures (of any material) which attach two membranes together are susceptible to relocation within the plane of the membranes when the membranes in which they are embedded are mechanically pulled apart (for example, by osmotic or freezing effects). In my work, I have found instances where such effects appear to be operating. Figure 1*f* is a particularly striking example of unfixed and non-cryoprotected myelinated nerve where it is believed that growth of ice has created quilt-like arrays of particles on the axolemmal P-face. These arrays are not seen in material which has been fixed. Arrays of this sort are believed to be indicative of structures which are visible in the plane of fracture and which link two membranes together. The location of tight junctions, gap junctions and other structures which link two membranes together may also be susceptible to relocation into clusters and quilt-like patterns as proposed.

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Visualization of genetic recombination intermediates of human adenovirus type 2 DNA from infected HeLa cells

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The study of recombination in prokaryotes has been facilitated by the availability of recombinational mutants and simple genetic elements such as phages and plasmids. These small but defined molecules of DNA have been especially useful for electron microscopic analysis of structural detail of molecules undergoing recombination both *in vivo* and *in vitro*¹⁻³. A limitation in the structural analysis of plasmid recombination is the absolute number of recombining molecules which can be identified and analysed amidst a background of nonrecombining molecules³. This limitation would be of even greater consequence in studies of genetic recombination in animal cells. We therefore chose virus-infected animal cells as a model system for the study of the molecular mechanism of genetic recombination in higher organisms. HeLa cells infected with adenovirus serotype 2 (Ad-2) offer several advantages for studying recombination: (1) the virus contains a small and well characterized genome of about 35 kilobases; (2) a large amount of Ad-2 DNA is accumulated during lytic infection and host DNA synthesis is suppressed⁴; (3) Ad-2 recombines at a very high frequency^{5,6}; and (4) similar to phages, animal viruses and Ad-2 in particular are believed to use many of the host cell's enzymes in necessary metabolic processes, presumably including recombination. In this study we used electron microscopic techniques to visualize the structures of *in vivo* Ad-2 DNA recombination intermediates. Molecules were observed with structures at putative cross-over points which were consistent with the molecular mechanism of recombination proposed by Holliday⁷. In addition, we observed Ad-2 DNA molecules engaged in recombination which were simultaneously serving as templates for replication and/or transcription. To the best of our knowledge, this is the first visualization of *in vivo* recombination intermediates of discrete DNA molecules isolated from eukaryotic cells.

Nucleoprotein complexes were isolated from Ad-2 infected HeLa cells late in infection (20–25 h), a time when viral DNA synthesis and un packaged viral DNA are maximal⁴. To minimize the cellular DNA background, Ad-2 nucleoprotein complexes were selectively extracted from purified nuclei by the procedures of Wilhelm *et al.*⁸ as described in the legend to Fig. 1 and in ref. 9. Aliquots from the sucrose density gradient-fractionated Ad-2 nucleoprotein complexes were prepared for electron microscopy as described by Davis *et al.*¹⁰. At 20 h post-infection the majority (~70%) of the molecules observed were found to be duplex unit-length Ad-2 DNA. Molecules engaged in viral DNA replication, transcription, and simultaneous replication and transcription were observed and are discussed in detail elsewhere⁹.

A small fraction (<1%) of molecules were observed which were characterized by the intimate association of what appeared to be two complete molecules of Ad-2 DNA. Such molecules were followed along their entire lengths under high magnification (~45,000 $\times$), photographs were made, and lengths measured to determine the nature of this association. Typical molecules are shown in Fig. 1. Two unit length molecules of Ad-2 DNA are joined at a region of sequence homology (as judged by position in the genome) wherein a cross-over

structure can be seen (Fig. 1, inset). Such structures, clearly distinguishable in ~10% of the identified recombination intermediates, were most probably generated by a local denaturation in the region of cross-over³ allowing visualization of the configuration of the participating strands. This configuration would be predicted from a Holliday-type model of recombination⁷ and has been visualized previously with plasmid DNAs³. These molecules were believed to represent true

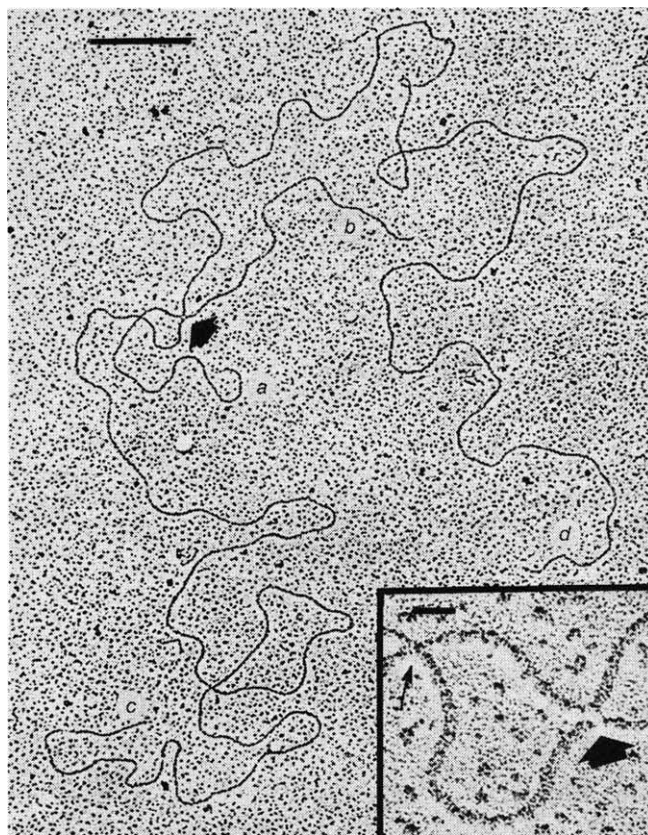


Fig. 1 Electron micrograph of a recombination intermediate of two molecules of Ad-2 DNA. Ad-2 nucleoprotein complexes were selectively extracted from Ad-2 infected HeLa cell nuclei by a modified procedure⁹ of Wilhelm *et al.*⁸. Twenty to 24 h post-infection, the cells were pelleted and resuspended in TITE buffer (20 mM Tris, 100 mM NaCl, 0.02% Triton X-100, 2 mM EDTA pH 7.4) and homogenized in a tight fitting dounce at 0°C. The homogenate was layered on 10% sucrose in TITE buffer and centrifuged for 5 min at 500 r.p.m. in a Damon IEC-6000 centrifuge. The pelleted nuclei were resuspended in TEAD buffer [20 mM Tris, 2 mM EDTA, 50 mM (NH₄)₂SO₄, 0.1 mM dithiothreitol, pH 7.9] and lysed by adding, drop by drop, 2 M (NH₄)₂SO₄ to a final concentration of 0.2 M. The lysate was allowed to stand for 10 min on ice and then was centrifuged for 10 min at 10,000g. The resulting supernatant or S200 was either layered directly onto 10–60% sucrose gradients in TEAD buffer⁸ or was treated with 0.25% sarkosyl and resolved in a 15–30% sucrose gradient containing 10 mM potassium phosphate, pH 7.9, 2 mM EDTA, a 0.25 to 0% sarkosyl gradient and 0.25% glutaraldehyde⁹. Centrifugation was in an SW40 rotor at 4°C for 17 h at 25,000 r.p.m. Various fractions from the sucrose gradient were analysed by electron microscopy. This particular preparation was spread in 50% formamide as described previously¹⁰. The cross-over point in the recombining molecules (large arrowhead) is enlarged in the inset on the lower right. The thin curved arrow in the inset indicates an overlap between strands which is clearly distinguishable from the Holliday-type structure indicated by the large arrowhead. The length of arm *a* (1.65 μ m) is equal to arm *b* (1.64 μ m), and *c* (10.68 μ m) is equal to *d* (10.60 μ m). The sum of *a*, *b*, *c* and *d* is 24.57 μ m which is equal to two unit-length Ad-2 genomes (the average length of the intact Ad-2 DNA molecules illustrated in this study is 11.82 $\pm$ 0.41 μ m). Scale bars, 0.5 μ m large micrograph and 0.05 μ m inset.

Fig. 2 Electron micrographs of Ad-2 molecules with Holliday-type structures at various distances along the genomes. Each photograph shows a close-up of the Holliday structure (arrow) and one of the two pairs of equal length arms. In (A), arm x is equal to y and represents 8.5% of the genome length, a distance closer to the ends than in the molecules shown in Fig. 1 where the Holliday structure lies about at 13.5% of the genome length. In (B), x is equal to y and the Holliday structure is located at a point about 29.8% from an end of AD-2 genome. Preparations were made as described in Fig. 1 legend. Scale bars $0.2\ \mu\text{m}$ (A) and $0.4\ \mu\text{m}$ (B).

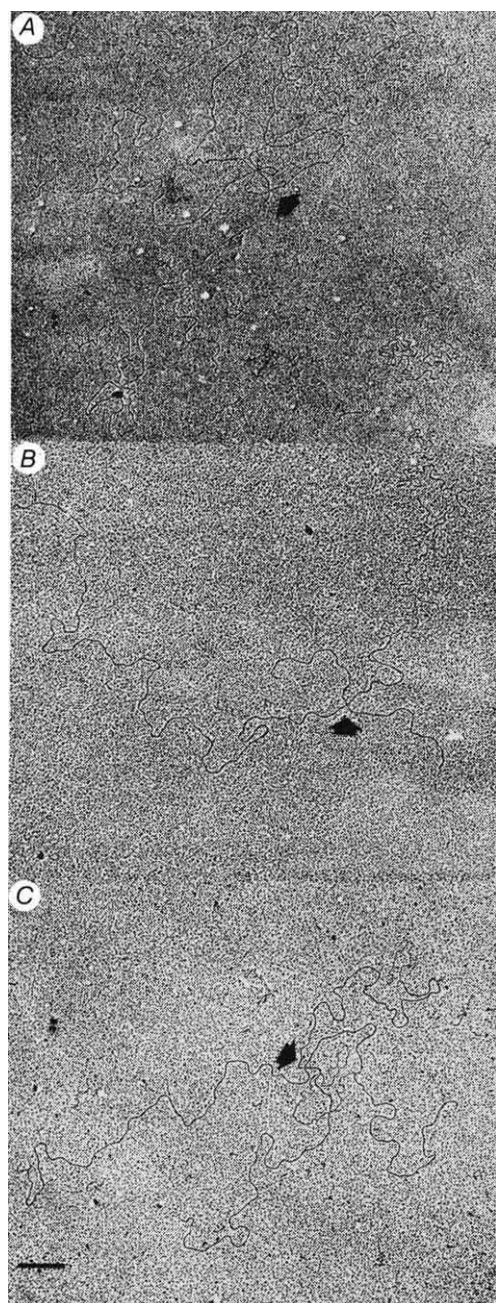
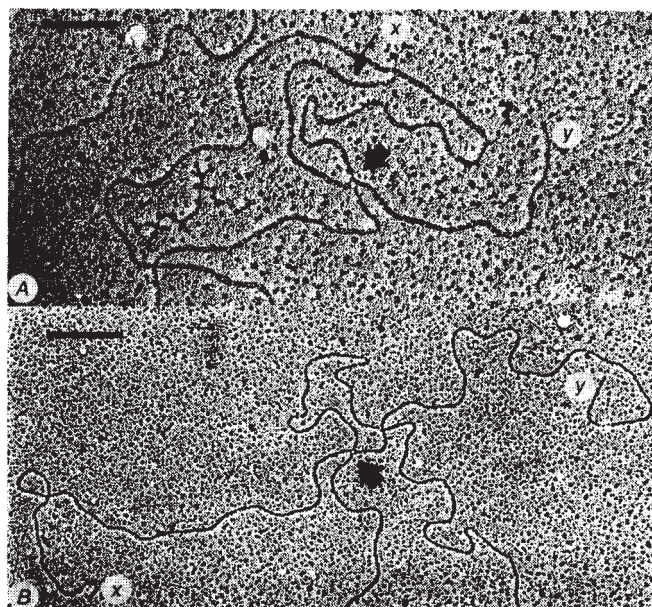


Fig. 3 Electron micrographs and interpretive tracings of Ad-2 recombination intermediates simultaneously engaged in DNA replication. The large arrowhead in each photograph and tracing indicates the point of putative cross-over. Double-stranded DNA is indicated by the solid black line; single-stranded DNA by the dashed line. A, Recombination involving an intact double-stranded unit length genome and a type I replication intermediate. The single-stranded displaced parental strand (dashed line) can be seen in the upper right. The sum of the four double-stranded arms is equal to two complete Ad-2 genomes. B, Recombination between an intact double-stranded Ad-2 molecule and a presumptive type II intermediate which is partially single-stranded and partially double-stranded. The cross-over point is between double-stranded regions of the two molecules. C, Recombination between two molecules containing both type I and type II replicative molecules. The sum of the three completely double-stranded arms and the one double-stranded and single-stranded arm is equal to two Ad-2 genome lengths. The single-stranded branch in the centre of the photograph represents the displaced parental strand complementary to one of the strands in the arm indicated as X. Cross-over junctions are located 41%, 16% and 46% along the genome lengths in A, B and C, respectively. The Holliday-type structure is visible in A but not in B or C. Scale bar, $0.5\ \mu\text{m}$.

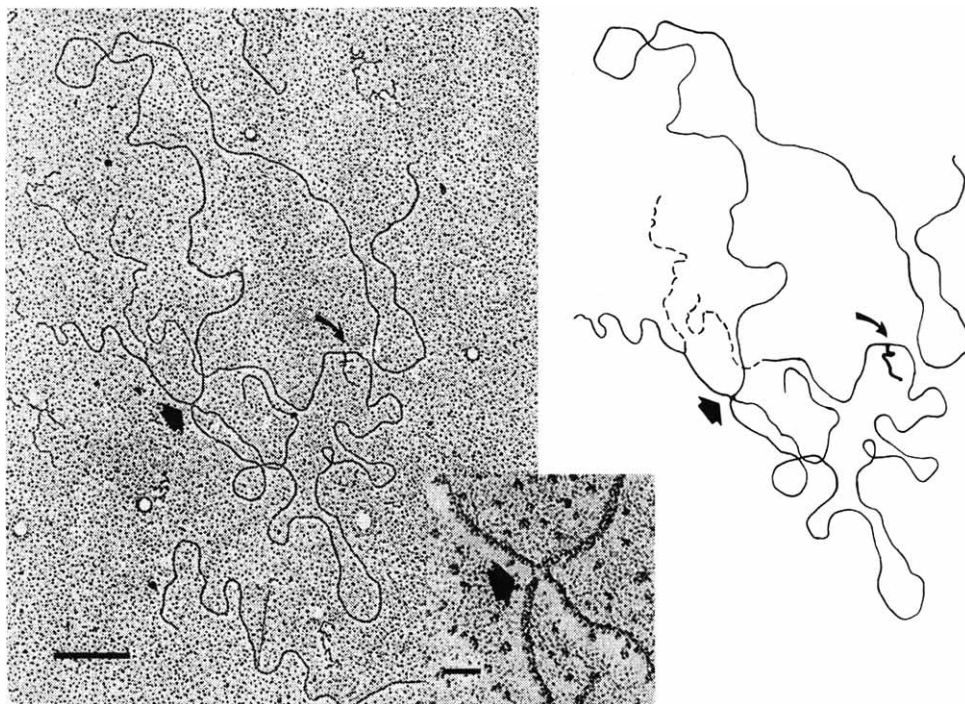


Fig. 4 Electron micrographs and interpretive tracing of recombination intermediate between replicative and transcriptionally active Ad-2 DNA molecules. The putative cross-over point shows a Holliday-type structure (indicated by the large arrowhead and enlarged in the inset). The curved arrow and heavy black line indicate a nascent RNA transcript (discussed in detail in ref. 9). Both type I and type II replication intermediates are seen. The single-stranded portions of these molecules are indicated by dashed lines; the double-stranded portions by the solid lines. Scale bars 0.5 μm large photomicrograph, and 0.05 μm inset.

recombination intermediates according to the following criteria: (1) the structure contained two sets of arms of equal length ($a = b$ and $c = d$ in Fig. 1) from the point of cross-over; (2) the sum of the lengths of the four arms was equal to two unit-length Ad-2 genomes; (3) the highly characteristic appearance of the strands of the molecules at the recombination juncture was observed and these structures were clearly distinguished from mere overlaps (curved arrow, inset to Fig. 1); and (4) such structures were never observed between molecules of purified SV40 DNA added to the preparation. As further illustrated in Fig. 2, these points of putative cross-over were found at various regions of the genome.

Recombination intermediates in which portions of the recombining Ad-2 genomes were apparently replicating were also observed (Fig. 3). The structure of replicating molecules of Ad-2 DNA has been described previously (reviewed in ref. 11). Briefly, Ad-2 DNA replication proceeds in a 5' to 3' direction from either end of the duplex molecule, displacing one of the parental strands. The resulting replicating intermediate (type I intermediate) contains two double-stranded branches and one or more single-stranded branches equal to the length of one of the double-stranded branches. For the replication of the displaced parental strand, the predominant mode of synthesis involves initiation at the 3' end, generating an intermediate with single-stranded portions at one end of the molecule (type II intermediate). As can be seen in Fig. 3 (A–C), both types of replicative intermediates were found to be engaged in the recombination process. The recombination intermediate in Fig. 3A involves a unit-length double-stranded molecule and a type I replicating molecule with its characteristic fork and displaced parental strand. In Fig. 3B, a type II molecule and an intact unit length double-stranded molecule are involved in recombination. Both type I and type II replicating molecules are involved in the recombination event depicted in Fig. 3C. An additional category of recombining and replicating molecules was identified which was comprised of more complex structures consisting of recombination intermediates with single-stranded branches at the cross-over point. Analysis of these molecules, which may represent recombination events involving the displaced parental strand, analogous to the model for recombination proposed by Meselson and Radding¹², is under way. One further template activity was identified in molecules undergoing recombination: in addition to consisting of replicating molecules of both type I and type II, one of the recombining

molecules shown in Fig. 4 also carries an RNA transcript. It is clear that the recombination event does not involve a sequestered group of molecules in these animal cells as they are simultaneously available to DNA and RNA polymerases.

These observations constitute direct structural evidence for the physical associations between recombining molecules predicted from the model of genetic recombination proposed by Holliday⁷ and previously observed in prokaryotes³. A feature of this model is a reciprocal exchange between the two genomes with no requirement for DNA synthesis other than a repair-type. Our observation of recombination between non-replicating Ad-2 molecules (Figs 1 and 2) and between replicating molecules but not involving the displaced parental strand (Figs 3 and 4) lends further support to the Holliday-type model of recombination as one possible mechanism for accomplishing genetic recombination during Ad-2 lytic infection. We believe that this is the first time such *in vivo* recombination intermediate molecules have been visualized after isolation from animal cells. The formation of recombinant DNA intermediates between exogenous plasmid molecules in the presence of an extract from unfertilized *Xenopus laevis* eggs reported by Benbow and Krauss¹³ was generated totally *in vitro*. Since animal virus genomes are limited in the amount of genetic information they can carry, they are believed to utilize a variety of cellular products to accomplish metabolic events. Elucidation of the molecular mechanisms of recombination in animal viruses in somatic cells should yield information on the genetic recombination process that occurs during meiosis in germ cells.

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Indomethacin and inhibition of protein kinase reactions

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Indomethacin, an inhibitor of prostaglandin biosynthesis, is useful in studies aimed at understanding the metabolism and physiological function of prostaglandins¹. A recent report² showing that indomethacin at 10^{-7} M potently inhibits the cyclic AMP-dependent protein kinases (cAMP-PrK) from ileal mucosa in the presence or absence of cyclic AMP, suggests how indomethacin may antagonize prostaglandin action on ileal mucosa. It also suggests that indomethacin might be useful in studying the properties and functions of protein kinase reactions. Inhibitors of prostaglandin biosynthesis, such as sodium salicylate and acetylsalicylate, at concentrations near 10^{-2} M, have been shown to inhibit bovine diaphragm protein kinase only in the presence of cAMP, while stimulating it in the absence of cAMP³. We report here that complete inhibition of cAMP-PrKs by indomethacin requires a concentration of 10^{-3} M and is not tissue-specific, and that the effect of indomethacin is concentration dependent above 2×10^{-4} M for the cAMP-dependent, and above 10^{-3} M for cAMP-independent PrKs. These results contrast previous ones².

Cyclic AMP-dependent protein kinase was purified from rabbit ileal mucosa and rat liver cytosol by a modification of methods described earlier^{4,5}. Rabbits (~4 kg) were used as the source of ileal tissue. Ileae were rinsed with cold distilled water, and the mucosa was isolated by stripping it off the muscle tissue. Sprague-Dawley rats (weighing 250–300 g, from ARS/Sprague Dawley) were used as the source of liver tissue. Protein kinases I and II, and their catalytic subunit, were purified through DEAE-cellulose and hydroxyapatite steps. Peak I was eluted from DEAE-cellulose at less than 0.1 M NaCl, and peak II at 0.15–0.2 M NaCl. The activity ratios (minus cAMP/plus cAMP) of peaks I, II and the catalytic subunit in the presence of 2 μ M cAMP were 0.4, 0.5 and >0.9, respectively, for the rabbit ileum preparation, and 0.17, 0.33 and >0.95, respectively, for rat liver preparations. The ratio of peak I to peak II was 15:85 and 45:55 for rabbit ileum and rat liver, respectively. Each of the three activities was inhibited by the classical cytosol cAMP-PrK inhibitor⁶. A cAMP-independent liver nuclear protein kinase was purified using DEAE-Sephadex and partially (30%) dephosphorylated phosvitin (DPV) affinity chromatography (unpublished work). The enzyme had insignificant activity toward lysine-rich histones, but was very active towards DPV, casein and liver nuclear endogenous non-histone proteins (NHP) as substrates. It was neither stimulated by cAMP nor inhibited by the cAMP-PrK inhibitor. Indomethacin solution was prepared by titration with 0.1 M NaOH of an aqueous suspension of indomethacin until the solution was clear (final pH 9.0). The extinction coefficient of this solution was 16,200 and 6,300 at 260 and 319 nm, respectively. This suggests that indomethacin was stable in these experimental conditions. Experiments were also carried out with indomethacin dissolved

in ethanol; in this case an appropriate amount of ethanol was added in control reactions. The results obtained were not dependent on the method of preparing the indomethacin solution.

As shown in Fig. 1, indomethacin at 10^{-4} M had no significant effect on any of the rabbit ileum mucosa cAMP-PrKs in the presence or absence of cAMP. Concentrations below 10^{-4} M were equally ineffective (data not given). The same result was

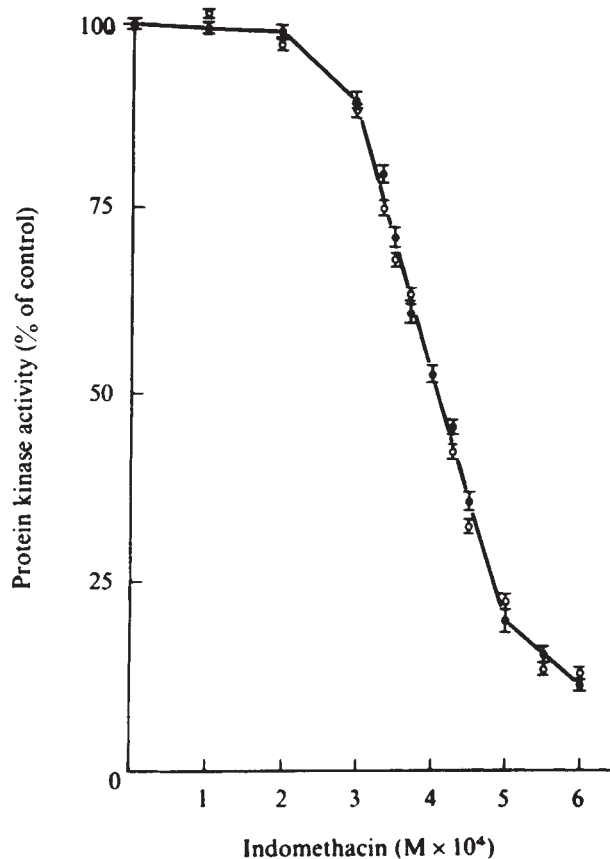


Fig. 1 Effect of varying concentrations of indomethacin on cAMP-PrKs from rabbit ileum mucosa, in the presence or absence of cAMP. The standard reaction medium for assaying cAMP-PrK was Tris-HCl, pH 7.45 (30 mM, 37 °C); LRH or protamine sulphate (1–5 mg ml⁻¹); MgCl₂ (10.0 mM); [γ -³²P]ATP, (0.2 mM, 30–50 μ Ci μ mol⁻¹); with or without cAMP (2 μ M); and, enzyme protein (0.04–0.2 mg ml⁻¹) in a final volume of 0.25 ml. The enzyme was allowed 20–45 min prior contact at 37 °C with all the reactants except MgCl₂ and [γ -³²P]ATP which were added together to initiate the protein kinase reaction, following which aliquots (40 μ l) were removed at 0, 4, 8 and 12 min. This material was rapidly spotted on P-81 paper, and the radioactivity incorporated in protein substrates was determined as described by Corbin and Reimann¹². The assay medium for cAMP-independent nuclear protein kinase consisted of Tris-HCl, pH 7.45 (30 mM, 37 °C); DPV (2 mg ml⁻¹); MgCl₂ (4 mM); [γ -³²P]ATP (3 mM, 2–5 μ Ci μ mol⁻¹), in a final reaction volume of 0.25 ml. The details of incubation were the same as described above. The amount of radioactivity incorporated in DPV was determined as described by Goueli *et al.*¹³. Each data point represents a mean of 10 experimental values $\pm$ s.e.m. for each tested enzyme; ●, plus cAMP; ○, minus cAMP. Statistical analysis using Student's *t*-test between activity values in the presence of 0, 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} M indomethacin showed no significant differences at 1% level. All protein determinations were according to Bensadoun and Weinstein¹⁴ using BSA, DPV and lysine-rich histones as standards. In some experiments α -glycerophosphate (pH 7.4, 37 °C) was used in place of Tris-HCl (pH 7.45, 37 °C) as buffer in protein kinase assays in the presence or absence of indomethacin. Change of buffer did not alter the results described above.

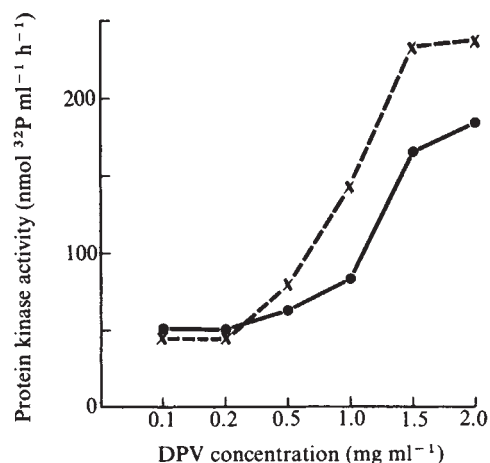


Fig. 2 Effect of varying concentrations of DPV on cAMP-independent nuclear protein kinase activity in the absence or presence of 10^{-3} M indomethacin. The details of assay were as described in Fig. 1 legend. ●, No indomethacin; x, 10^{-3} M indomethacin. Each point represents a mean of three different values $\pm$ s.e.m.

obtained with a number of different enzyme preparations, for example liver protein kinase peaks I, II and the catalytic subunit, bovine heart protein kinase peak II (data not shown). Also, the order of addition of various reactants, or the duration of prior contact of the enzyme with the drug, was without effect: the incubation of enzyme with the drug for up to 45 min at 30°C, or 20 min at 37°C, gave the same results as when no prior contact between drug and enzyme was allowed. Addition of 1 mM dithiothreitol had no effect on the protein kinase activity either in the presence or absence of indomethacin. When indomethacin concentration was increased to 10^{-3} M, all of the cAMP-dependent enzymes tested were inhibited, in the presence or absence of cAMP. These findings are in contrast to the previous report² which showed that inhibition by indomethacin was time dependent and maximal at a concentration of 10^{-7} M. Only at concentrations of indomethacin of 2×10^{-4} M or higher was there an inhibition of the ileal mucosa enzyme, as well as enzymes from other tissues. The concentration of the inhibitor required for 50% inhibition of the enzyme⁷, ID_{50} , for the ileal mucosa enzyme was 6×10^{-4} M, and similar values of ID_{50} were obtained when enzymes from other tissues were tested. We also examined the endogenous incorporation of 32 P into cAMP-PrKs. Again, at concentrations of indomethacin above 10^{-4} M, the pattern of inhibition was similar to that noted when protamine or lysine-rich histones were used as substrates (data not given). Thus, our results suggest that indomethacin is not a potent inhibitor of cytosol protein kinase reaction and that the inhibitory effect is not specific for any of the kinases examined. This is similar to the observation that sodium salicylate at 10^{-2} M inhibited the kinase activity by 50% but was without effect at 10^{-3} or less³.

The effect of indomethacin on a nuclear cAMP-independent protein kinase active toward DPV was also studied. We found that indomethacin did not inhibit this particular reaction, even at a concentration of 5×10^{-4} M; rather, a small stimulation (20–25%) of the kinase activity was observed in these conditions (data not shown). However, at higher concentrations (for example at 10^{-3} M), a small inhibition (25%) of the enzyme activity became apparent. Thus, it appears that this cAMP-independent kinase activity is even more insensitive to indomethacin than that dependent on cAMP. Furthermore, when liver chromosomal NHP was used as substrate for the cAMP-independent PrK reaction, the pattern of inhibition by indomethacin was similar to that observed with DPV as substrate (about 25% inhibition was noted at 10^{-3} M indomethacin).

To determine whether or not substrate concentration modified the indomethacin effect in this reaction, we tested the action of the drug on nuclear protein kinase activity at different concentrations of DPV in the reaction. Figure 2 shows that in the presence of 10^{-3} M indomethacin, this protein kinase reaction was only slightly inhibited in the presence of DPV at a concentration of about 0.2 mg ml^{-1} . However, at higher substrate concentrations (0.5 – 2.0 mg ml^{-1}) a stimulation of the kinase activity was noted. The above results suggest that indomethacin binds to DPV (a model acidic protein substrate) and lysine-rich histones or protamine (basic protein substrates) in a manner that influences their activity as substrates. It appears that the drug renders a more favourable conformation to DPV and a less favourable conformation to histone or protamine in the respective protein kinase reactions. This is somewhat analogous to the observed *in vitro* effects of polyamines on various protein kinase reactions in the presence of acidic or basic protein substrates⁸. However, note that binding of the drug to the enzyme itself (presumably with low affinity) to produce these changes cannot be excluded. The avid binding of indomethacin to plasma proteins has been reported; over 90% of indomethacin in the blood is bound to plasma proteins and only 10% is free for therapeutic effect¹. To test whether or not indomethacin binding to proteins in the reaction was reversible, we added 2 – 10 mg ml^{-1} of bovine serum albumin (BSA) to the reaction in the presence and absence of indomethacin. BSA had no effect on the activity in the absence of indomethacin, but in its presence it completely reversed the inhibitory effect of 10^{-3} M indomethacin on cAMP-PrKs, suggesting that the indomethacin inhibition was reversible (data not shown). This is also in contrast with the previous conclusion² on the nature of indomethacin interaction with the enzyme.

The reason for marked differences between our results and those of Kantor and Hampton² is not clear. However, we have used a number of different sources of the kinases which have yielded similar results. The general properties of these enzymes are analogous to those reported in the literature. It is well known that cytosol protein kinases from a variety of cells behave similarly; the only variation is demonstrated in the ratio of peaks I and II without any significant differences in enzymatic properties between peak I enzymes isolated from different tissues^{9,10}. Thus, our results indicate that the indomethacin effect is not specific for any of the cAMP-PrKs, regardless of their source, and that complete inhibition of all of the enzymes requires 10^{-3} M concentration of the drug. Similarly, a variety of other enzymes is also known to be inhibited by indomethacin at concentrations in the range of 10^{-5} – 10^{-4} M (ref. 11). Such effects of indomethacin, apparent only at higher concentrations, may contribute to the toxic manifestations of indomethacin, rather than reflecting a mechanism of therapeutic action *in vivo*¹¹.

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BOOK REVIEWS

Human behaviour: do the genes have it?

David P. Barash

In his collected *Letters*, John Keats once wrote:

I go amongst the fields and catch a glimpse of a stoat or a field mouse peeping out of the withered grass. The creature hath a purpose and its eyes are bright with it. I go amongst the buildings of a city and I see a man hurrying along — to what? The creature hath a purpose and its eyes are bright with it.

We may derive two inferences from Keats's poetic prose: (1) living things have a purpose, and (2) purpose is shared by human beings and animals. Unlike poets, who delight in it, scientists typically are trained to run the other way when 'purpose' rears its head. Nonetheless, this seemingly unscientific djinn keeps popping up in modern evolutionary biology. And, thanks to W.D. Hamilton's updating of Darwin, we are finally groping toward an understanding of that purpose: maximization of inclusive fitness. It is becoming increasingly clear that living things go about their business in a way that projects a maximum number of their genes into succeeding generations. Better yet, that *is* their business — and hence their purpose. (Alternatively, Richard Dawkins has emphasized that perhaps it is really the genes who are running the show.)

Like Wilson's *On Human Nature*, this book by Richard Alexander argues that inclusive fitness maximization is a philosopher's stone for understanding human behaviour. It fits well into the current sociology of sociobiology. For some reason, sociobiology has emerged primarily as a US–Britain concord, while continental Europe still contributes largely to ethology rather than its neo-Darwinian cousin. In addition, the British school has maintained a deafening silence on *Homo sapiens*, whereas the Americans are increasingly turning their attentions to the human species. Both groups, however, admire inclusive fitness. Indeed, sociobiology seems to have passed the stage of Thomas Kuhn's paradigmatic revolution and entered into the more extended phase of 'puzzle solving' or 'normal science'.

Alexander's book has its quirks: in 317 pages the word sociobiology is not once volunteered (signs of alpha male–male competition within the field). His prose is often turgid, and his presentation elaborate and convoluted. For example, we are served up Alexander's critique of

Darwinism and Human Affairs. By Richard D. Alexander. Pp. 317. (University of Washington Press/Pitman: 1980.) \$14.95, £9.50.

anthropologist Marshall Sahlins's denial of Alexander's interpretation of Sahlins's work on reciprocity in primitive societies. Sometimes, however, he rises to near-poetry:

Happiness . . . is eating and sex and parenthood and warmth and touching and ownership and giving and receiving and loving and being loved; it is the cessation of pain and the onset of pleasure; it is finding a way to win; it is having a magnificent idea or a grandchild.

But Alexander isn't Keats, and biology is never far away: "Happiness is a means to reproduction".

Human sociobiology is more than just inclusive fitness theory, but regrettably, we find no mention of male–female differences and parental investment theory, or of parent–offspring conflict. Even sexual selection, a current rage among most sociobiologists, receives short shrift. Although he is at pains to clarify that sociobiology does not imply genetic determinism, Alexander seems naively untouched by the vigorous and specific political objections that others have raised. In the conclusion of his *Origin of Species*, Darwin rather diffidently suggested that "light will be shed" on human beings as well. With light often comes heat. Some of the recent heat deserves considering, if only to refute it: for example, because of its emphasis on the biological unity of *Homo sapiens*, human sociobiology is a potentially potent antidote to racism.

Alexander has steeped himself in anthropology and the results are rewarding: both data and theory on cross-cousin marriage as an adjunct to incest avoidance, more original material on the mother's brother phenomenon, documentation on the overwhelming importance of kin and the curious phenomenon of self-deception, which the author suggests may help explain the reluctance of many to accept the findings of sociobiology applied to themselves. In many ways, this work is a *tour de force*, repeating and elaborating Alexander's many cogent arguments and original presentations of the past few years. He suggests a provocative view of justice, in which "laws function to place limits on

reproductive striving", and an ingenious if somewhat disillusioning approach to right and wrong. His usually tight reasoning is less convincing, however, in a concluding treatment of the rise of nation-states, a subject to which evolutionary biology seems to offer little beyond debatable, *post hoc* interpretations.

Homo sapiens is undoubtedly the best-known and most widely studied species on our planet. And yet, ironically, the data base from which sociobiological propositions can be tested is extraordinarily unsatisfying. Alexander relies heavily on G.P. Murdoch's cross-cultural samples and upon the Human Relations Area File. As one who desires to paint in broad strokes on a world-wide canvas, he has no alternative, although anthropologists are (or should be) painfully aware that the HRAF especially is a nightmarish potpourri, ranging unpredictably from careful, quantitative documentation to mere anecdote. It is all we have, however, and therefore its use is justified. But nonetheless, numbers gleaned from such a source derive a reified exaltation that they only rarely merit. In human sociobiology, as in any other science, conclusions are only as robust as the data and, except in recent studies (often conducted specifically to test evolutionary hypotheses), these data tend to be woefully inadequate.

Nonetheless, human sociobiology comes to upgrade social science, not to replace it. Julian Huxley once warned biologists against "nothing butism", the notion that because we are animals we are necessarily nothing but animals. The warning is well taken, but can be turned around with equal force: just because human behaviour is largely the product of social learning and cultural tradition, we should not assume that our behaviour derives from "nothing but" these experiences. We are special, but we are also animals nonetheless, and as in most dichotomies the truth doubtless lies somewhere in between.

Critics are justified in rejecting "just so stories", and to his credit Alexander is especially adroit at establishing falsifiable hypotheses. Animal studies should not be uncritically extrapolated between species, and certainly this includes human beings. However, research on animals can provide propositions about the manner in which natural selection appears to act upon

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behaviour, even complex social behaviour. Then these predictions — based ultimately on fitness maximization — can be tested cross-culturally. Thus, the world's human societies constitute a global experiment, with many varying cultures and one constant: our biology. The occurrence of universals such as nepotism and incest avoidance therefore suggests some biological underpinnings. Moreover, if these universals coincide with the predictions of fitness maximization, then social scientists ought to take notice. If biology seems arrogant in seeking to include human behaviour within its view, then what of the greater arrogance of those claiming no such help is needed, or wanted?

As the work of Alexander and others is increasingly showing, much of what human beings actually do accords remarkably well with the prediction of what we expect them to do if they are maximizing their inclusive fitness. But what in turn does this mean? It seems unlikely that we possess genes 'for' discrete cultural phenomena, but equally unlikely that culture and biology are totally uncoupled. Accordingly, new and productive directions may well emerge from current work such as that described in the forthcoming book, *Gene-Culture Evolution* (Harvard University Press), by C. Lumsden and E. O. Wilson.

The real question is not: Does evolution say anything about our behaviour?, rather: How much does it say? Since evolutionary theory is 'right', then human sociobiology cannot possibly be wrong — but it can be misleading. Maybe non-adaptive evolution has had a larger say than most sociobiologists typically assume — maybe

fitness is only rarely maximized and optimality theory is just a convenient mythology. Whereas Darwin's message seems indisputable, Hamilton's is less so, although it certainly seems correct. And much of sociobiology (and nearly all of Alexander's work) relies on Hamilton's inclusive fitness theory. Finally, maybe human behaviour is just too dependent on complex non-genetic processes of social learning and cultural experience to be much elucidated by selfish DNA molecules jousting with each other to get ahead. But we owe it to ourselves to find out.

Physicist Jean Perrin once wrote that the goal of science was "to explain the complicated visible by some simple invisible", and Einstein suggested similarly that "the ground aim of all science is to cover the greatest number of empirical facts by logical deductions from the smallest possible number of hypotheses". Our own behaviour is a complicated visible, providing an overwhelming array of empirical facts. Sociobiology employs a simple and powerful invisible (evolution by natural selection), based on a seductively unitary hypothesis (differential reproduction of individuals and their genes). When the wife of the bishop of Worcester first heard of Darwin's scandalous new theory, she is reported to have cried "Descended from monkeys? My dear, let us hope that it isn't true! But if it is true, let us hope that it doesn't become widely known!" By contrast, let us hope that the implications of evolution do become widely studied, for *Homo sapiens* no less than any other species. □

David P. Barash is Professor of Psychology and Zoology at the University of Washington, Seattle.

Inspiration for particle physicists

Maurice Jacob

Models of High Energy Processes. By J. C. Polkinghorne. Pp. 131. (Cambridge University Press: 1980.) \$24.95, £11.

HIGH energy physics is a fast-moving field, and the paucity of specialized textbooks bears witness to the fact that any detailed exposition of a particular topic runs the risk of becoming rapidly obsolete or of covering a field which drops from the forefront of research. This slim volume cleverly avoids such a predicament. Its discussion of specific techniques will make it a useful tool for many years to come. Indeed, the detailed consideration of how Feynman diagram techniques can be used to obtain asymptotic properties and specific behaviours of model amplitudes covers

topics which were of great interest to particle physicists in the 1960s (Regge behaviour) and 1970s (parton model and perturbative quantum chromodynamics) and which are likely to relate to important new developments. The book should be a source of inspiration to the graduate student in theoretical physics and also rewarding to the more knowledgeable reader, for whom it provides access to the wide range of applications of Feynman graph techniques. A combination of heuristic arguments and detailed mathematical treatment makes it easy reading, for reference or as a textbook.

It should be mentioned, however, that, despite its title, this book does not provide a full survey of models of high energy processes. The selective list of topics covered clearly reflects the research interests of the author and his preferred lines of approach. At the same time, many topics are not introduced or discussed as thoroughly as many readers, and in particular students, might wish to see. Mere reference to specialized books will

often be inadequate for subjects which are no longer well known to the newcomer, such as Regge behaviour. A more detailed discussion of some topical questions (in particular those involving perturbative QCD) would have been useful to bring the reader fully abreast of present applications. One should, however, mention a detailed presentation of the parton model in a field theoretic language which has recently provoked

much interest.

Despite its being too short to meet all requirements, this little book should certainly provide the reader with ideas and with a better understanding of present approaches to high energy interactions. □

Maurice Jacob is a senior scientist in the Theoretical Study Division of CERN and a corresponding member of the French Académie des Sciences.

Hypoxia as a way of life

R. D. Harkness

Living without Oxygen: Closed and Open Systems in Hypoxia Tolerance. By Peter W. Hochachka. Pp.181. (Harvard University Press: 1980.) \$17.50, £10.50.

THE thing that strikes you immediately about this book is that the author is an enthusiast. And not simply an enthusiast, but one whose enthusiasm has led him to bring together a great diversity of material to make a coherent story. There is a point in everything he has written, and that, is an unusual achievement for a work that is primarily about biochemistry, a subject prone to accumulation of disconnected facts.

The book is concerned with the ways in which animals obtain energy when they have limited or no access to oxygen. For some this is part of their way of life (intestinal parasitic worms, deep-sea fishes); for others it is a temporary state (fishes wintering in stagnant water, lungfishes sealed in dried mud, bivalve molluscs with their shells shut, seals and other marine mammals when diving). The same sources can provide, for rapid movement, a short-lived burst of energy at a higher rate than could be achieved with the use of oxygen alone (vertebrate and cephalopod muscle). This latter mechanism by degradation of carbohydrate (glycogen) to lactic acid, will be familiar to many who would, like myself, be ignorant of other things dealt with in this book. However, the production of lactic acid has snags — its acidity which can adversely affect other tissues. The effect is reduced in diving mammals by use of the acid in organs privileged to have what little oxygen is available, principally the brain and heart (and also, curiously, the adrenals). The problem can also be solved by the production of less awkward end-products, such as by creatures with particular adaptation. One, curiously, is alcohol, produced by goldfish under conditions where little oxygen is available. The fish make ethanol by using in reverse the same set of enzymes as we do to destroy it. how much they enjoy their grog we don't know,

but it is nice to think of them quietly solacing themselves as they lie in winter in the mud at the bottom of a cold pond waiting for the spring; and maybe solace others too as they get rid of the alcohol into the surrounding water.

There is much more like this on the curious adaptations which have evolved to enable animals to operate in unusual environments made interesting by the fact that the author is not only a biochemist but also a zoologist. This is what really makes this book so fascinating — he puts the biochemistry into the whole picture of the physiology and life-style of the animals.

So this is a good book which should certainly be in any library that caters for degree students. Is there, then, nothing wrong or the lack of something that would make it better? The approach is primarily biochemical, and the reader needs to be familiar with both the general concepts and the facts of biochemistry. For example, no chemical formulae of compounds are given, only names; enzymes are shortened to abbreviations, explained it is true at the first mention, but not easy to find if you want to check; there is no glossary. Yet the book will interest many who are unfamiliar with biochemical terms, and a short general summary of the biochemical background would be of great value to the non-specialist. To argue that the information is available elsewhere is to miss the point that it is not available when and where it is wanted. Written by Professor Hochachka, at the start of the book, it would not only be in the right place but also probably clearer and more pertinent than anything one could easily find elsewhere.

In one of my excursions into history I came across William Robertson, a most readable 18th century man, who habitually started you off a few hundred, if not thousands, of years earlier. So it would be nice if this book could start in the same sort of way to make it more easily understandable, possibly not to historians, but to a range of people who would find it very interesting but at the moment rather a struggle. So, in summary, an alpha now, and every prospect for an alpha plus for the next edition. □

R. D. Harkness is Professor of Physiology at University College London, whose main interest has been in connective tissue with a bias towards comparative physiology.

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Clouds in the interstellar medium

B. E. Turner

Giant Molecular Clouds in the Galaxy.
Edited by P. M. Solomon and M. G. Edmunds. Pp. 300. (Pergamon: 1980.)
£18, \$41.

THE distribution and physical properties of the neutral interstellar gas in our galaxy have been major subjects of investigation since the discovery of interstellar atomic hydrogen in 1951. With the discovery 12 years ago of interstellar molecules as a new investigative tool, these studies have been extended to the molecular component with the undertaking of two major surveys of galactic carbon monoxide (CO) in 1975. The present volume is a collection of papers on this and related subjects as they stood in mid-1977 at the Third Gregynog Astrophysics Workshop, held at the University of Wales.

As with much of astrophysics, our perceptions of the interstellar medium vary on a timescale rather short compared with that of the publication of this volume. Even the title suffers as a result. It refers to one of two main topics covered, namely the properties of the molecular cloud ensemble that is generally agreed to comprise by mass at least 75% of the total gas of the galaxy interior to the Sun. The principal question is whether the cloud ensemble is dominated by relatively few 'giant molecular clouds' (GMCs) or by a larger number of smaller clouds. Curiously, the title belies the fact that only one of the two survey groups (Solomon and his collaborators) subscribe to the notion of GMCs, while the other group (Burton and Gordon) find that smaller clouds dominate. Loosely, GMCs may be defined as having masses in excess of $10^5 M_{\odot}$ (diameters > 40 pc) as distinct from the many smaller clouds (10^2 – $10^4 M_{\odot}$) seen optically near the Sun. Whether or not GMCs dominate (the question is one of interpretation, not of differences in survey data by the two groups) is important to our understanding of galactic structure, because relatively few GMCs affect the distribution of stars and their motions much more than does the same quantity of gas residing in more numerous, smaller clouds. In the past year, extensive modelling of galactic cloud distributions by Liszt and Burton has shown that the observations are consistent with an ensemble of numerous, smaller clouds with at most only about 4% of the clouds by number being GMCs. Although the title of the book may therefore describe only the minority component, it must be emphasized that GMCs are known observationally, the dozen or so examples being discussed in the first two papers.

Star formation, and its association with molecular clouds, is the other principal

topic of this book. Where stars may be directly observed, it has been clear for some time that massive OB stars form in or at the edges of the larger molecular clouds (mass of $10^4 M_{\odot}$ or larger) while only low-mass stars (including T Tauris) form in small clouds. Observational examples of each of these cases are given in two papers. Other articles discuss various mechanisms for the onset of massive star formation, such as density wave implosions, sequential formation by O star radiation, supernova inducement, cloud-cloud collisions and so on. Doubts about the applicability of any of these mechanisms to at least one object (CRL961) are discussed by L. Blitz, who contributes much of the observational material in this book not available in the open literature. The probable association of OH and H₂O masers with massive protostellar objects is set forth in three observational papers, and the relation of HII regions and molecular clouds is the topic of two papers. On the theoretical side, magnetic fields (the *bête noire* of any model of star formation) are discussed with originality if inconclusion in two papers.

In addition to the above two topics, this volume includes several articles on peripherally related subjects: some outdated work on the galactic centre (two papers); two works on highly specific aspects of interstellar chemistry (on nitrogen chemistry and interstellar biochemicals — the latter is notably speculative and has appeared in journals several times); and finally, even an entertaining paper on the search for planetary systems.

As might be judged from such a plethora of topics, the book is not intended, nor does it succeed, as a synthesis of any aspect of interstellar clouds. Unfortunately, there are no discussion sessions which might have tied the various viewpoints together; thus the forum of a workshop is not reflected in the book. The introduction by the editors serves as a (rather one-sided) overview, but not more.

The belated publication of this volume has limited the usefulness of much of it; no fewer than 21 of the 31 papers, and parts of some others, have appeared in journals. Nevertheless, some valuable material has not been published elsewhere. Examples are the beautiful colour maps of the Solomon *et al.* CO survey, the large-scale CO maps of the Cygnus X, Taurus and Perseus regions in the paper by Blitz, and several of the theoretical discussions.

The book is handsomely produced on high-quality paper, with fine illustrations and a well-thought-out index. Overall, it is certainly to be recommended as a reference book for institutional libraries, and at the price it may well find a place in the personal collections of serious students of the interstellar medium. It is less suitable for a more general audience. □

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